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ASPECTS OF RESPIRATION IN *PLANORBIS CORNEUS* L. AND *LYMNAEA STAGNALIS* L. (GASTROPODA: PULMONATA)*

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(*Received* 27 *June* 1961)

Abstract—1. *P. corneus* and *L. stagnalis* were compared in respect of various aspects of diving behaviour; variation in volume and composition of the pulmonary air during the dive; simultaneous pulmonary and cutaneous O_2 uptake as a function of dissolved pO_2 (measured by an original method which is fully described).

2. The variation of dissolved pO_2 in the natural habitat was studied.

3. In both species the lung is of considerable importance even when dissolved pO_2 is as high as 220 mm and becomes increasingly important as the tension falls. The lower limit of pulmonary pO_2 is 2·8 per cent in *Planorbis*, 8·8 per cent in *Lymnaea*.

4. The hypothesis is advanced that haemoglobin in *Planorbis* facilitates greater exploitation of the pulmonary O_2 store and so permits a deeper and longer dive compared with *Lymnaea*, which lacking a respiratory pigment occupies a separate ecological niche nearer the surface.

INTRODUCTION

THE fresh-water pulmonate, *Planorbis corneus* L.† has been the subject of a number of investigations aimed at elucidating the function of its respiratory pigment. The reports of these studies sometimes draw attention to the supposed fact that "the presence of haemoglobin in the invertebrates has generally been found to be correlated with a habitat at times deficient in oxygen" (Borden, 1931). Leitch (1916) makes an almost identical statement, but without the qualifications. These workers further suppose that *Planorbis* particularly well exemplifies this correlation and their studies were designed to test, and purport to show, the value of the haemoglobin in sustaining aerobic metabolism in oxygen deficient surroundings. Perhaps, because of this pre-judgment of the issue, subsequent work has been concerned mainly with the oxygen-binding properties of the haemoglobin. Comparatively little attention has been paid to the question of respiratory gas-exchange and to the nature of the normal respiratory conditions.

Leitch (1916), Mačela & Seliškar (1925), Wolvekamp (1932) and Fox (1945) have all found that the affinity of *Planorbis* haemoglobin for oxygen is high.

* This work was carried out in the Dept. of Experimental Zoology, Zoological Laboratory, University of Leiden, during the tenure of a scholarship awarded by the Netherlands Ministry of Education, Arts and Sciences.

† Since no other species of the genus is anywhere referred to in this paper, the generic name alone is used subsequently. Similarly '*Lymnaea*' should always be understood to refer to *L. stagnalis* L.

A more recent study by Zaaijer & Wolvekamp (1958) not only confirms the high oxygen affinity but examines very thoroughly the influence of temperature and pH. There can be no doubt that the properties of *Planorbis* haemoglobin are such that it ought to be very useful in oxygen transport when the pO_2 of the arterial blood is low. Both Leitch (1916) and Borden (1931) concluded from estimates of total haemoglobin content and respiration rate that the 'O_2-storage' value of the pigment was unimportant.

The question remains to be answered, whether the pigment does in fact function as an oxygen transporter and at low ambient tensions only. Leitch's paper includes some observations which appear to indicate that it does. She observed the behaviour of normal and carbon monoxide treated snails in waters of different oxygen tensions. Only when the pO_2 was below 25 mm did the two groups show any difference in regard to the frequency of rising to the surface to replenish the air in their lungs. Although this points to a pigment function solely at low pO_2 in the conditions of her experiment, it means little in relation to the normal life of the animal. Her experimental arrangement was such that the fall in dissolved oxygen tension was paralleled by a similar fall in the gas-phase from which the animals refilled their lungs; this circumstance is hardly likely to be found in nature.

Studies of respiratory gas-exchange have been made by various workers but our knowledge on this point cannot be regarded as satisfactory. Borden (1931) found a mean oxygen consumption of 26 ml/kg/h, with a wide individual variation, by shaking very small snails (*ca.* 0·5 g) in a Barcroft differential manometric apparatus. Jordan (1930) reported experiments carried out by some of his students, without giving any details of methods or of the number and distribution of the original data. Thus in a comparison (by Hazelhoff) of *Planorbis* and *Lymnaea stagnalis* the oxygen content of the lung, on arrival at the surface to refill, was 4·1 and 13·7 per cent respectively. The time course of oxygen depletion in the lung was also found to be different. In *Lymnaea* the per cent O_2 fell rapidly in the first hour, very slowly thereafter, while in *Planorbis* the fall was uniform. In another study reported without experimental details, it was found (by Fournier & Bunschoten) that rate of oxygen uptake from the lung of *Planorbis* was almost constant when the initial pulmonary pO_2 was between 120 and 40 mm. Above and below these levels the uptake was very pressure dependent.

More recently Füsser & Krüger (1951) have made a very painstaking comparison of the oxygen consumption of *Planorbis* and *Lymnaea*. Most of their measurements were of total oxygen consumption of snails in water-saturated air. There was a very pronounced variation in respiration rate for both species but the mean rate for *Planorbis* in air with 21 per cent O_2 was only 60 per cent of the rate for *Lymnaea*. Oxygen consumption was strongly pressure dependent for both species down to about 40 mm, though the curve fell less steeply for *Lymnaea*. Between 40 and 10 mm pO_2 the oxygen consumption of *Planorbis* appeared to remain constant while that of *Lymnaea* fell more sharply than before. When 5 per cent carbon monoxide was added to the gas mixtures the oxygen consumption of *Planorbis*

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was unaffected unless pO_2 was greater than 120 mm or less than 40 mm. Surprisingly, however, in the case of *Lymnaea* which lacks a respiratory pigment, the effect of CO was to depress very drastically the oxygen consumption at all ambient oxygen pressures.

Zaaijer & Wolvekamp (1958) found that there was no appreciable difference between *Planorbis* and *Lymnaea* in the ability to deplete the available dissolved oxygen, when the snails were confined in water without access to air.

In order to put discussion of the function of *Planorbis* haemoglobin on a more secure footing, it seemed desirable to make an investigation of a number of aspects of respiratory gas-exchange under conditions approximating those found in nature. For sake of comparison all observations were duplicated with *Lymnaea*. This snail resembles *Planorbis* in many ways both as regards anatomy, respiratory behaviour and habitat but lacks a respiratory pigment. In view of the anomalous results obtained by Füsser and Krüger with carbon monoxide and for other reasons which have been discussed elsewhere (Jones, in press) the carbon-monoxide method offers a very uncertain means of elucidating pigment function. It was felt that a comparison between similar species with and without pigment might provide an alternative approach to this problem. The present paper presents results of a preliminary survey of some aspects of the question.

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METHODS

General

Dissolved oxygen was determined according to the micro-Winkler technique of van Dam (1935) except that phosphoric acid was used to liberate the iodine from the reaction mixture. In the gas exchange experiments the oxygen uptake from the water was calculated from the change in concentration of dissolved oxygen in a known volume of water during a given time.

In order to give a complete account of the pulmonary gas exchange it is necessary to determine both the composition and the volume of the air in the lung. A method for the measurement of the volume of the lung air in a rapid and simple manner, compatible with the determination of the other necessary factors, is the principal technical innovation in the present work and a full description is given below. It is not sufficient to determine the oxygen uptake from the lung simply by measuring the change in the size of the air bubble even if, as is theoretically probable, metabolic carbon dioxide is almost entirely taken up by the water. Such a simple gasometric approach is invalidated by the fact that as oxygen is absorbed from the lung the total pressure of the bubble remains the same, within limits set by the vertical movement of the animal in the water, and accordingly, the partial pressure of nitrogen will rise and this gas will also tend to be absorbed. For a full discussion of this topic the paper of Wolvekamp & Vlasblom (1952) should be consulted.

The analyses of samples of air from the lung were carried out in the micro-gas-analyser of Krogh (1908), according to his procedure but with the minor modifications previously described (Jones, 1959).

Simultaneous Determination of Aerial and Aquatic Gas Exchange

The principle adopted for the measurement of pulmonary air volumes rests on a simple application of Boyle's Law. The animal is sealed in a respiration chamber which is otherwise entirely filled with water and which is designed so that the total volume of the system can be conveniently reduced by an accurately measured amount. The ensuing change in pressure can be read on a mercury manometer and the volume of the compressible gas phase, which is confined to the pulmonary air, can be calculated or read off from a calibration curve.

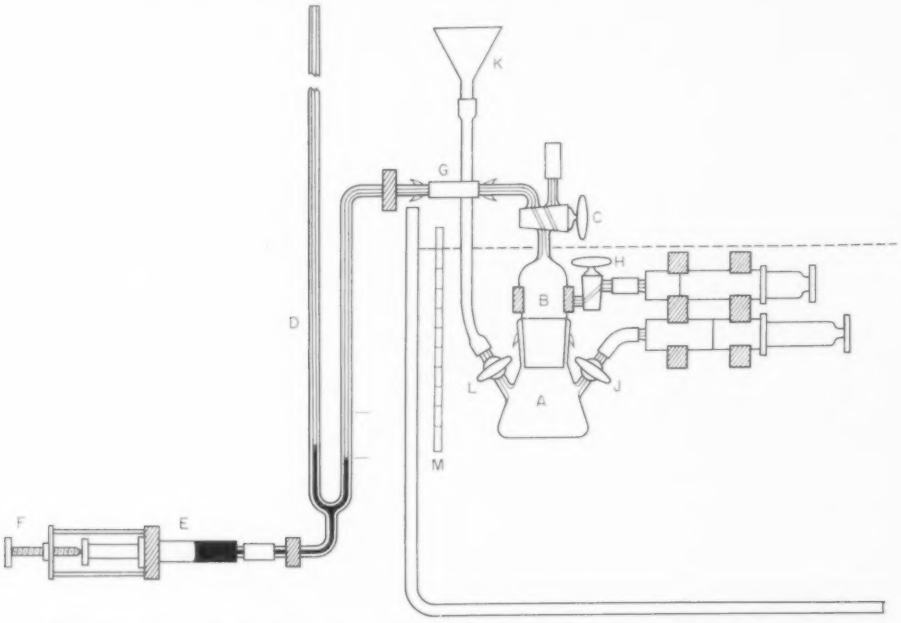

FIG. 1. Gas-exchange apparatus designed for simultaneous measurement of pulmonary and cutaneous oxygen uptake. Cross-hatched areas represent position of clamps which hold the components firmly to a single vertical sheet of 'Perspex'. Both arms of the manometer are graduated in mm; the two marks against the right limb indicate the mercury levels before and after effecting the standard volume reduction.

A diagrammatic representation of the apparatus is given in Fig. 1. The respiration chamber consists of a thick-walled conical flask A of about 100 ml capacity and a hollow stopper B. These are connected through a standard cone and socket joint (B.34) wide enough to allow unhindered passage (between the stopper and the flask) of snails weighing up to 4 g. The two-way tap C connects the stopper (a) with the outside for the withdrawal of water samples by means of a syringe-pipette and for the introduction of air or (b) with the mercury manometer D on which both volume and pressure changes are registered. The space

between the mercury in the right-hand manometer arm and tap C is filled with water. At the bottom of the manometer tube there is an outlet connected by thick-walled plastic tubing to a syringe E which acts as a mercury reservoir. The syringe plunger is held firmly by rubber bands against the adjusting screw F which is used to effect the volume and pressure changes. The manometer arms are of uniform bore capillary tubing with a diameter of about 1 mm, and are graduated in mm. The right-hand arm is accurately calibrated by weighing mercury delivered from various parts of the scale. In order to simplify cleaning and reduce the fragility of the apparatus, the manometer is not fused to the stopper. Instead there is a joint G at which the two ends of the tubing are ground flat and held firmly in contact with each other by tightly fitting plastic tubing and a pair of springs. Oblique-bore taps H and J are connected by plastic tubing to a pair of 10 ml syringes. Reciprocating movement of these syringes produces very thorough mixing of the water in the chamber, which is essential if the Winkler analyses are to give a reliable measure of the uptake of dissolved oxygen. These syringes also permit volume compensation for withdrawal of water samples and enable air to be drawn into and expelled from the stopper when the animal rises to fill its lung. The funnel K and oblique-bore tap L serve for filling the apparatus with water. The cone and socket joint is sealed by means of a P.T.F.E. plastic sleeve and secured by means of strong rubber bands passing over the two-way tap, to and from the hooks on the flask. Within the apparatus a positive pressure of 20 cm Hg can be maintained without leakage. All parts of the apparatus except the flask are mounted rigidly on a vertical sheet of perspex (·8 cm) standing on three feet; the sheet has a vertical slot running up from the lower edge so that the flask, stopper and syringes can be immersed to the indicated level in a constant temperature water-bath while the manometer and its adjusting syringe remain outside.

The joint G and the connection between tap H and the upper syringe, once set up without air bubbles, can remain so unless it is necessary to dismantle for cleaning. Prior to each experiment it is necessary only to make good the cone and socket joint and to connect the lower syringe. The volume of the apparatus including the various connecting tubes and the dead spaces of the syringes should be determined to $\pm 0{\cdot}5$ ml. In the present case it amounted to 132 ml.

In setting up the apparatus for gas exchange observations the flask is filled with water of the required oxygen tension and the animal is placed in it. With tap C open to the air, the cone and socket joint is carefully but firmly closed with a twisting action to get a good seal with the plastic sleeve, care being taken to avoid trapping any air bubbles at the base of the cone. The joint is secured by means of the rubber bands. Tap J is opened and any air bubbles in it are displaced before the lower syringe, filled with water, is connected. This syringe is adjusted to the 5 ml mark and tap J closed. The remaining air in the apparatus is displaced by admitting water from the funnel K and the upper syringe is allowed to fill (without air bubbles) to the 6 ml mark. When the whole apparatus has been checked for the exclusion of air bubbles it is placed in the water-bath and allowed

to come to temperature equilibrium. The water-bath temperature was regulated, by means of a mercury contact thermometer, to 15 $\pm$ 0·02° C.

The subsequent procedure for one set of measurements of simultaneous oxygen up-take from the lung and from the water is as follows:

(a) When the animal rises into the stopper an air space of about 5 ml is introduced through tap C by withdrawing the upper syringe. As soon as the animal has filled its lung the air is displaced and tap C closed.

(b) With all taps closed, except H, the plunger of the upper syringe is pulled out to lower the internal pressure sufficiently for a small part of the pulmonary air bubble to be extracted. This rises to the top of the stopper and by opening tap C and pressing on the syringe plunger the bubble is quickly transferred to the cup of the micro-gas-analyser. The latter has previously been clamped with the cup in contact with the water-filled plastic tube on the outlet of tap C. Part of the bubble (*ca.* 0·005 ml) is drawn into the analyser capillary without delay and there may remain for subsequent analysis.

(c) Tap C is turned to connect with the manometer and, tap H being open, the mercury level is adjusted to a standard zero in the right-hand limb by means of the screw F. The level in the left limb is noted and the depth of the animal below the level of the water-bath surface is roughly determined with reference to the measuring stick M at the moment of closing tap H. By means of the screw F, the mercury level in the right limb is raised precisely to a second standard point 3 cm above the zero and the new level in the left arm is noted. Atmospheric pressure is restored by re-adjusting the screw.

(d) Tap C is closed and, with taps H and J open, the water in the chamber is thoroughly mixed by a reciprocating action of the syringes. The lower syringe is adjusted to 5 ml and tap J closed. The tip of the van Dam syringe-pipette, with its dead space filled with water, is pressed firmly against the rubber seal above tap C, which is then opened. The plunger of the syringe-pipette is worked in and out three or four times and finally a water sample of known volume (*ca.* 0·5 ml) is retained. The upper syringe is adjusted to the 5 ml mark and then tap C is closed. Tap H remains open so that the pulmonary bubble remains at approximately atmospheric pressure throughout the subsequent respiration period.

(e) The composition of the bubble in the micro-gas-analyser is now determined and the normal Winkler–van Dam procedure carried out on the water sample.

(f) At the end of the desired observation period the above steps are repeated with the difference that the final pulmonary air volume is determined (c) before a sample is extracted for analysis (b). During the observation period the water in the chamber is gently mixed by working the syringes periodically.

In using the apparatus according to the above scheme the following points should be noted. With care the portion of air removed from the lung in step (b) does not exceed $\frac{1}{10}$th of the total volume. It is important, however, that the whole of this portion be removed from the chamber and this is readily done so

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long as none of the extracted air is trapped under the rim of the shell. This latter eventuality is best avoided, in the case of *Planorbis*, by waiting until the animal is moving head downward from the top of the stopper before reducing the pressure; this is the normal behaviour after filling the lung. No difficulties were found in this respect with *Lymnaea*. If any air bubble becomes apparent during the course of an observation period, that set of observations must be discarded. The animal should not be allowed to reach the flask before the initial air extraction, otherwise bubbles may become lodged under the rim formed by the base of the cone. For the final air extraction these precautions are unnecessary.

In carrying out the determination of the pulmonary air volume [step (c)], the pressure should be raised only for as long as is strictly necessary, in order to avoid excessive gas absorption from the lung.

If desired, a second observation period can begin immediately after the animal has been allowed to refill its lung, sufficient water having been added to the apparatus to maintain the standard water volume (volume of apparatus, less volume of animal, plus 10 ml in the syringes). It is not absolutely necessary to maintain such a standard volume but if this is adopted as part of the routine it helps to obviate errors in the dissolved oxygen-uptake calculations through omitting to note the syringe volumes after step (d). Finally, after releasing the animal from the chamber its volume is approximately determined by displacement and its weight recorded after drying with filter paper.

For each observation period one has, therefore, data from which initial and final values of (a) dissolved oxygen concentration, (b) pulmonary air volume and (c) pulmonary air composition can be calculated. The simplest method of deriving pulmonary air volumes from the pressure data is to construct an empirical calibration curve. This relates observed pressure increases (for the standard volume change) to the known volumes of calibration air bubbles introduced by withdrawal of accurately measured volumes of water from the apparatus. However, in testing the apparatus it seemed desirable to try to relate observed pressure values to theoretical ones. In doing so, three points emerged which must be accounted for if volumes are calculated.

(i) With tap H open the pressure of any gas within the apparatus is B.P. plus the pressure of the water above it. Calibration bubbles lying immediately below tap C are at a constant depth, but pulmonary air will be at a pressure somewhat dependent upon the depth of the animal below the surface of the water bath. This depth should be approximately measured immediately before closing tap H for volume determination and this can be conveniently done by having a rod (M) suspended in the water bath beside the apparatus, which is marked at intervals of 13·5 mm ($\equiv$ 1 mm Hg). This correction, designated C in the formula below, will be as much as 9 mm for an animal at the bottom of the flask. Pulmonary volumes whether determined by calculation or from a calibration curve are related to the pressure $(B+C)$ and should be corrected to standard pressure before calculating the pulmonary oxygen-uptake.

(ii) The increase in pressure p, resulting from the standard volume reduction, is equal to the rise of the mercury level in the left limb less the difference between the mercury and water pressures equivalent to the rise in the right limb; i.e. less (30 mm Hg – 30 mm H_2O) if the standard volume reduction is represented by a 3 cm rise in the right limb. This may

not be immediately apparent but can be demonstrated by taking moments about the level of the standard zero before and after raising the pressure.

(iii) The movement of the mercury in the right limb may not be entirely a representation of the compression of the gas-phase, but may in part reflect elasticity of the apparatus. In this case the volume reduction of the gas-phase (v) will be somewhat less than the volume equivalent of the mercury rise in the right limb. This can be easily corrected by making an elasticity curve. In the original apparatus there was a slight but consistent elasticity in the flask. This was allowed for by plotting an elasticity curve in the form relating corrected pressure increase p to corrected volume change v; the latter was obtained by deducting the volume equivalent of the rise in the right limb for particular values of p determined when the apparatus was entirely filled with water.

Due allowance being made for the three points discussed immediately above, a very close agreement was found between the actual volumes of air bubbles accurately measured into the apparatus and the volumes calculated according to the formulation below.

X = actual volume of pulmonary air at moment of closing tap H (ml)
v = true change in volume of pulmonary air due to increase of pressure (ml) [see note (iii)]
B = barometric pressure (mm Hg).
C = pressure due to water above the lung at closing tap H (mm Hg) [see note (i)]
p = pressure increase, corrected (mm Hg) [see note (ii)]

According to Boyle's Law the conditions before and after raising the pressures may be equated:

$$X\,(B+C) = (X-v)\,(B+C+p)$$

Whence

$$X = v(B+C+p)/p$$

As mentioned above (i), values of X (determined at pressure $B+C$) must be corrected to standard pressure before calculating pulmonary oxygen uptake. This calculation simply involves finding the difference between the oxygen contents (per cent O_2 × volume) of the initial and final bubbles. Pulmonary oxygen consumption is this oxygen uptake corrected for time and weight.

Materials

Specimens of both species were collected from a shallow drainage ditch about 4 km north of Leiden during October 1959. They were housed in small aquarium tanks of about 2 l. capacity in a basement room in which the air temperature was controlled at 15 ($\pm 0{\cdot}5$)°C. The tanks were illuminated for 12 h/day by means of 60 W fluorescent tubes about 30 cm above the water surface. On alternate days the snails were fed on a calcium alginate gell, containing wheat germ and dried milled lettuce. Under these conditions most of the *Planorbis* specimens survived for over eight months before being returned to the ditch. Throughout this period they remained active and laying viable eggs. Those which died probably succumbed to the trematode infection which was widespread amongst them, since they suffered haemorrhages before withdrawing into their shells in the manner described by Wesenberg-Lund (1939).

The above-mentioned conditions were less satisfactory for *Lymnaea*, which did not survive longer than three months; no obvious reason for this mortality could be found. All the recorded observations on this species were made on specimens collected from the original spot in May and June 1960. These fresh

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animals were nevertheless housed in the standard basement conditions for at least 3 weeks before use.

Two or three days before use for any respiration experiments, specimens were transferred to an aquarium suspended in the respirometer water-bath, which was continuously controlled at 15°C. In order to avoid the possibility of post-prandial variations of the respiration rate, such as were reported by Berg & Ockelmann (1959), all animals were starved overnight before use.

It is worth noting that the first batch of snails brought into the laboratory died in 2 days and it was subsequently established that this was due to copper poisoning. The animals had been placed in water from a tap fed by copper piping. Thereafter only water from an old part of the building, drawn through lead and iron pipes, was used and no further difficulties were encountered. This problem had not arisen in maintaining other fresh-water animals (notably a wide variety of fishes) in the new building aquaria.

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RESPIRATORY BEHAVIOUR

Patterns of behaviour may have an important bearing on respiratory gas exchange, especially in aquatic animals. While neither of the present species exhibits such a strikingly regular behaviour pattern as is frequently met with in tubicolous animals, a general description must be attempted. In addition certain aspects have been subjected to simple quantitative assessment and experimentation.

General description

Both species visit the surface periodically in order to replenish the supply of air in the lung. This is accomplished by protruding the markedly hydrophobe margin of the mantle into the surface film, the margin being thrown into an almost complete circle in order to form a short temporary 'siphon' leading into the mantle cavity. The duration of this lung-opening is very variable and if less than 20 sec, the opening will often be repeated before the animal leaves the surface. The normal duration is considerably longer than this and periods exceeding 2 min have been observed occasionally in both species. During the open period, the volume of gas absorbed in the preceding dive will be replaced and to a greater or lesser extent, dependent on the duration and extent of the opening, diffusion will restore the composition of the pulmonary air towards that of atmospheric air. It will be seen later that in neither species does the pO_2 of the pulmonary gas reach the atmospheric level; the deficiency is significantly greater in *Planorbis*. Nothing has been seen, even in young specimens in which the soft parts can be seen through the transparent shell, to suggest that there are any ventilation movements.

After closing the lung the animal normally dives and only returns to the surface for the next lung-filling. However, in this respect there is a difference between the two species. In *Planorbis* this diving behaviour is very deliberate, both in nature and in aquaria, the animals rarely remaining at or even near the surface except when filling the lung. In *Lymnaea* the movement away from the

surface is less deliberate and individuals can often be seen creeping on the underside of the surface film. Yet the lung remains closed during this surface creeping until a deliberate opening occurs after a normal interval.

It has frequently been observed, both in nature and in an artificial ditch in the laboratory, that *Planorbis* tends to be active on the bottom mud during dives and uses the plants principally as a means of access to the surface. *Lymnaea* on the other hand is not often seen on the bottom but spends its diving periods creeping about on the plants.

Under laboratory conditions, including those of the artificial ditch, individuals of both species periodically withdraw into their shells and remain inactive, sometimes for several hours. This periodic withdrawal is more frequent in *Planorbis*; to what extent it occurs in nature is not known. At temperatures between 5 and 10°C *Planorbis*, in an aquarium with mud on the bottom, bury themselves but emerge from time to time, at intervals of an hour or so, to refill the lung, after which they return to bury themselves once more. Wesenberg-Lund (1939) reported that at temperatures below 5°C, *Planorbis* buried itself in the mud and remained in a state of hibernation.

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Duration of the dive

One aspect of respiratory behaviour which can be measured very simply in the laboratory is the frequency of lung-filling or the duration of the dive. In Leiden, attempts to discover whether variation in dissolved oxygen concentration has any influence on this frequency had given negative results. Since it had been demonstrated that the rate of oxygen uptake from the lung increases with falling concentration of dissolved oxygen (see below), this matter was reinvestigated in Sheffield under somewhat more rigorously controlled conditions.

The animals had been acclimatized to a temperature of 18°C and to a constant level of fluorescent tube illumination (12 h/day) for three weeks and the experiments were carried out under the same conditions. A pair of animals of each species was placed in each of three 1 l. jars containing well-aerated pond water and allowed to feed during the night preceding the observations. In the morning the jars were refilled with water (at 18°C) whose oxygen content had been adjusted to one of the chosen levels. No food was given during the observation periods which commenced $\frac{1}{2}$ h later. In order to minimize gas-exchange with the atmosphere the water was not stirred; samples for Winkler analysis were drawn from the lower part of each jar at the beginning and end of the observation periods. The duration of the dive was taken as the interval between one lung-filling and the next, provided that during this period the animal made a deliberate move away from the surface. This criterion excluded very short intervals which sometimes occurred with *Lymnaea* when the animal remained at the surface after lung-filling. Also excluded were any intervals which included periods of withdrawal into the shell, an occasional occurrence with *Planorbis*.

One *Planorbis* was consistently inactive on each of the 3 days of observations and one *Lymnaea* died between the 2nd and 3rd days. Intervals recorded for

either of these animals were excluded from the final tally. The combined mean intervals for the remaining five animals of each species are given in Table 1. Interspecific differences are not significant, but differences as between the different oxygen tensions are significant except for the medium and high levels in the case of *Lymnaea*.

TABLE 1—DURATION OF DIVES (MIN) AT DIFFERENT DISSOLVED OXYGEN TENSIONS*

pO_2	285 mm	$\frac{d}{\sigma_d}$	115 mm	$\frac{d}{\sigma_d}$	28 mm
Planorbis	64·6 ± 6·5 (25)	3·1	37·9 ± 5·8 (26)	4·3	19·8 ± 1·3 (68)
Lymnaea	48·9 ± 9·3 (28)	1·8	33·6 ± 3·1 (41)	4·7	19·3 ± 1·4 (61)

* Each mean and S.E. relates to a number (in brackets) of dives executed by five animals; same five animals of each species observed at each pO_2. Since the variation for each individual animal was similar to that of the whole group, means and S.E. have been calculated on the basis of the total number of dives at each pO_2. The values of d/σ_d relating to the adjacent means on either side are given; d/σ_d values for interspecific differences 1·4 or less.

The influence of buoyancy on diving behaviour

The values obtained for the composition of the pulmonary air at the end of a diving period (see below) do not give any indication that a critical level of either pO_2 or pCO_2 is responsible for terminating the dive. On the other hand, it was often noted that, in the early stages of a dive, the shell and its contents were distinctly buoyant, while at the end of a dive animals appeared to be able to support this burden only with a deliberate effort. This state of affairs was most readily seen when an animal was moving more or less horizontally along a vertical or near vertical surface.

A simple arrangement was adopted to investigate the possibility that a loss of buoyancy of the shell (which would normally result from the absorption of part of the pulmonary gas) is a contributory factor in terminating the dive.

A stout 500 ml conical flask was provided with a tightly fitting stopper which could be clamped in position. The flask was filled with water to the bottom of the neck and one or two snails were introduced. Three tubes passed through the stopper from the air space above the water. One of these connected with a mercury manometer and another opened to the air through a stopcock. The third connected with the top of a glass bulb partly filled with air and partly filled with mercury. Through a connection at the bottom of the bulb, the mercury level could be adjusted by raising and lowering a reservoir. With this apparatus a snail could be subjected, as desired, to an excess pressure of up to 1 atm. Thus at any time the pulmonary air volume could be diminished by as much as 50 per cent.

The animal having been allowed to fill its lung with air at barometric pressure and dive to the bottom of the flask, the pressure was raised by one atmosphere.

The pressure was allowed to return to normal as soon as the animal, returning to the surface, reopened its lung. When it reached the bottom once more the pressure was again raised, and so on.

The behaviour of animals subjected to such conditions is best recorded and illustrated by plotting their positions (depth) in the flask in relation to time, as in Fig. 2. In general it was found that with both *Planorbis* and *Lymnaea*, the response to raising the pressure within a minute or two after lung-filling was a

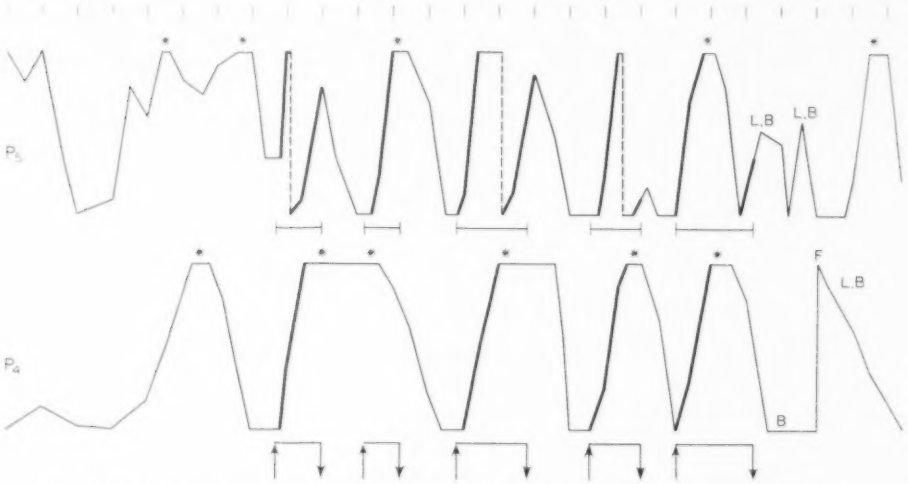

FIG. 2(a). Activity record for two specimens of *Planorbis* subjected simultaneously to pressure changes as described in the text; vertical movements of the animals as a function of time-intervals of 5 min on scale above. * moments of lung-opening; ↑ increase of pressure by 760 mm, ↓ return to B.P. Dotted lines indicate that the animal fell after losing its grip on the side of the flask. The thickened parts of the record distinguish upward movements made under increased pressure. Towards the end of the period P4 showed signs of abnormal buoyancy (B), having left the surface before decompression and later floated to the surface (F); shortly after this it lost bubbles from the lung (L.B.). P5 also lost bubbles from the lung following the last decompression.

more or less immediate return to the surface. Here the lung would be reopened at once, the interval between this and the previous filling being very much less than under normal conditions. Sometimes the immediacy of the response was apparently lessened because the animal, having reached the flat bottom of the flask, spent 1 or 2 min 'finding' the way up. The record reproduced in Fig. 2(a) is typical of those obtained with *Planorbis*. In this case two animals were observed simultaneously in the same flask and there is a remarkable synchronization of their upward movements. In a few *Planorbis* cases, the rise to the surface following pressure increase was not followed by any attempt to open the lung so long as the pressure remained high, but opening occurred immediately on partial decompression.

All but one of the series of *Planorbis* observations were made with atmospheric air in the space above the water, so that the premature termination of the dives in response to pressure increase was made in spite of the fact that the pulmonary pO_2 was not much less than twice the normal initial value. However, with one pair of *Planorbis* the space above the water was flushed through with a gas mixture containing only 10 per cent of oxygen, in order that the pulmonary pO_2 would

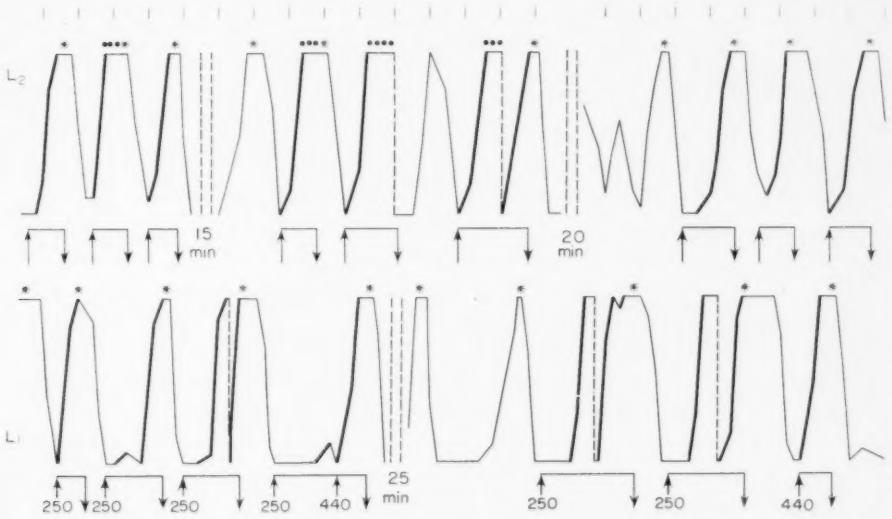

FIG. 2(b). Record of two separate observations on *Lymnaea*. Indications as in Fig. 2(a); dots above the record indicate unsuccessful attempts to open the lung. In the lower record smaller pressure increases were used as indicated below pressure change markers. Between the double dotted lines portions of the record (duration indicated) have been omitted; during these periods pressure remained at atmospheric and the animals made no approach to the surface.

be normal during the period of increased pressure. This pair of animals gave the most striking performance of all with five synchronized rises in a period of 25 min. It is interesting to note that although these particular snails were filling their lungs from an atmosphere which, while at atmospheric pressure, contained only 10 per cent of oxygen they showed no reluctance to commence their dives with an abnormally low pulmonary pO_2.

Essentially similar results were obtained with *Lymnaea*. In this species there was never any delay in opening or at least in attempting to open the lung on reaching the surface. Some individuals seemed to have difficulty, under an excess pressure of 1 atm, with consequent loss of buoyancy, in hitching the shell high enough to get the siphon to the surface. If, after one or two unsuccessful attempts, the apparatus was partially decompressed, no further difficulty was experienced.

A few observations were made with excess pressures of only 250 mm. This would result in only a 25 per cent reduction in pulmonary gas volume. *Planorbis* failed to make any clear-cut responses under these conditions but in most cases the response of *Lymnaea* was as already described though somewhat less prompt. The record of Fig. 2(b) illustrates the *Lymnaea* results.

Clearly a sufficient increase in the pressure (of the order of one atmosphere) has a positive influence on the diving behaviour of both species. A reasonable hypothesis would be to attribute this to a loss of buoyancy or increase of specific gravity of the shell complex, consequent upon the reduction of pulmonary gas volume. A comparatively simple proprioceptor mechanism would suffice to mediate the response.

Response to breathing pure N_2 *or pure* O_2

A few qualitative observations were made on animals confined in stoppered conical flasks in which the free space in the neck was gently flushed with cylinder N_2 or O_2 throughout the observation period. The gas-phase would not be quite pure because of a certain amount of exchange at the water surface.

In both species there was an immediate departure from the normal behaviour pattern on first opening the lung to the atmosphere of N_2. In every case, instead of diving on completion of a normal lung-filling, the animal remained at the surface and the 'siphon' was opened repeatedly at intervals of about 1 min. In *Planorbis* this 'gasping' continued on various occasions for 10, 30 or even 45 min. Eventually the animals dived, only to return within a few minutes for more repetitive lung-fillings. *Lymnaea* appeared somewhat less reluctant to leave the surface with an inadequate pulmonary pO_2, the longest 'gasping' period being 13 min, but again fresh excursions to the surface followed abnormally quickly. The oxygen concentration in the water was 6·5–5·0 ml/l. The first excursion to the surface, after restoration of a normal atmosphere, was marked by a single exceptionally long lung-opening, followed by a dive of normal duration. In this respect the behaviour of the two species was identical.

Observations on the response to breathing pure oxygen were fewer and the results less conclusive than in the case of nitrogen breathing. One specimen each of *Planorbis* and *Lymnaea* were found, during the course of two quite separate observation periods, to become completely inactive and withdrawn or extremely sluggish, respectively, for periods of more than 1 h after a single normal lung-opening to the O_2 atmosphere. The abnormal reaction was recognizable within 10 min of the exposure. One other specimen of each species, in the course of a single observation period, was apparently unaffected by breathing O_2, although in these cases the diving periods following exposure were abnormally long.

OBSERVATIONS ON RESPIRATORY GAS EXCHANGE

Initial and final composition of pulmonary air

In addition to those values obtained in the course of the complete gas exchange measurements (see below), a number of determinations were made specifically to

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establish the range of pulmonary air composition in animals housed in aquaria and also in their 'home' ditches. Specimens were intercepted at the surface immediately before or immediately after filling the lung and quickly transferred to a small dish of water. The animal, held below the surface just under the immersed cup of Krogh's micro-gas-analyser, was gently stimulated with a blunt probe until a portion of the pulmonary air was expelled. This was caught in the analyser cup and a suitable quantity was immediately withdrawn into the capillary.

TABLE 2—PERCENTAGES OF OXYGEN AND CARBON DIOXIDE IN PULMONARY AIR AT BEGINNING AND END OF DIVING PERIODS—MEAN AND S.E.*

	Venue	Initial Bubbles		Final Bubbles	
		Planorbis	*Lymnaea*	*Planorbis*	*Lymnaea*
O_2	Aquaria (18)	15·2 ± 0·51	19·0 ± 0·12	7·9 ± 0·84	10·2 ± 0·53
	Ditch (6)	16·2 ± 0·99	18·2 ± 0·48	4·2 ± 1·47	8·8 ± 0·81
	Apparatus (28)	15·0 ± 0·34	17·1 ± 0·34	7·2 ± 0·60	7·8 ± 0·42
CO_2	Aquaria (18)	2·0 ± 0·45	1·3 ± 0·14	1·9 ± 0·19	1·5 ± 0·12
	Ditch (6)	1·2 ± 0·19	1·1 ± 0·20	1·3 ± 0·19	1·1 ± 0·12
	Apparatus (28)	3·0 ± 0·31	1·7 ± 0·10	2·9 ± 0·37	2·1 ± 0·14

* Interspecific O_2 differences are significant in the case of initial bubbles from aquaria ($P<·01$), final bubbles from aquaria ($P= ·03$) and final bubbles from the ditch ($P= ·02$). In the case of the 'apparatus' figures the periods were not natural dives but the fixed observation periods described in the text. Figures in brackets indicate number of observations.

During this sampling procedure there may have been a slight loss of CO_2 to the water but the bubbles expelled from the lungs and trapped in the analyser cup were so large and the transfer to the capillary was so rapid that the exchange of O_2 with the water must have been negligible.

Table 2 gives, for both species, the mean initial and final percentages (beginning and end of diving periods) of O_2 and of CO_2 for animals in aquaria and in ditches. For purposes of a comparison which is discussed below, the percentages found at the beginning and end of observation periods in the gas exchange apparatus were also included.

Initial and final volume of pulmonary air

Determinations of initial and final pulmonary air volumes were also made separately from the observations on complete gas-exchange, but they were made, necessarily, in the gas-exchange apparatus, using one animal at a time.

The figures obtained for *Planorbis* are probably quite satisfactory, since the pattern of diving was clear cut. The animals moved to the bottom after filling their lungs and remained in the lower part of the flask for periods ranging from 35 to 91 min before making very deliberate movements up into the stopper. It was thus possible to know with some precision the correct moment for making the measurement of the final volume.

Lymnaea was a less satisfactory subject in this respect. Perhaps because its diving behaviour is, even under natural conditions, less clear cut, it was often difficult to judge the correct time for measuring the final volume. In the event, the diving period was regarded as complete when the animal had remained for two minutes in the top of the stopper. The duration of some of the 'dives' is

TABLE 3—INITIAL AND FINAL VOLUMES OF PULMONARY AIR (ml)

Planorbis					*Lymnaea*				
Snail wt. (g)	Duration of dive (min)	Initial Vol	Final Vol	% change	Snail wt. (g)	Duration of dive (min)	Initial Vol	Final Vol	% change
2·02		—	0·39		3·66	73	0·55	0·41	25·4
		0·43	0·39	9·3		55	0·63	0·50	20·6
		0·45	0·38	15·6		28	0·63	0·54	14·3
		0·49	—						
2·45		—	0·50		3·25	45	0·54	0·47	13·0
		0·52	0·47	9·6		67	0·56	0·46	17·9
		0·53	0·48	9·4			0·62		
		0·55	0·49	10·9	3·16	23	0·57	0·52	8·8
		0·58	0·49	15·5		40	0·62	0·55	11·3
		0·54	—			50	0·58	0·47	19·0
3·08		—	0·55			47	0·60	0·51	15·0
		0·63	0·60	4·8	3·46	70	0·56	0·43	23·2
		0·65	0·58	10·8		75	0·59	0·47	20·3
		0·71	—				0·69	—	
2·59	35	0·56	0·53	5·4		72	0·63	0·49	22·2
	91	0·60	0·54	10·0	3·48		—	0·41	
	40	0·57	0·53	7·0		22	0·53	0·46	13·2
	68	0·57	0·53	7·0		67	0·55	0·43	21·8
		0·58	—				0·51		
2·79		—	0·54			45	0·54	0·43	20·4
	40	0·59	0·53	10·2			—	0·45	
	90	0·60	0·54	10·0		19	0·52	0·48	7·7
		0·61	—			18	0·53	0·48	9·4
2·32	37	0·48	0·46	4·2		38	0·52	0·44	15·4
	75	0·51	0·48	5·9					
	56	0·50	0·47	6·0					
		0·53	—						

clearly much longer than was found in other contexts, so that some of the figures for final volume are undoubtedly too low and the figures for per cent change in volume are correspondingly too high.

The results of these volume determinations are set out in Table 3. Where the initial and final volumes for a particular dive were determined, the per cent change in volume has been computed. In addition, the pulmonary-air volume per gram

(total weight including shell) has been calculated for each snail on the basis of the respective mean initial volumes. These values are, for the individual animals, in the order in which they appear in the table: *Planorbis* 0·23, 0·22, 0·22, 0·22, 0·20, 0·22 ml/g; *Lymnaea* 0·17, 0·18, 0·19, 0·18, 0·15 ml/g.

TABLE 4—COEFFICIENTS AND STANDARD ERRORS OF THE REGRESSIONS OF TOTAL, PULMONARY AND CUTANEOUS O_2 CONSUMPTION ON DISSOLVED pO_2 (ARITHMETIC CO-ORDINATES) FOR *Planorbis* AND *Lymnaea**

	Planorbis	Inter-specific differ-ences	*Lymnaea*
Total	−0·01137 ± 0·02101 (1)		−0·01042 ± 0·01374 (1)
Pulmonary	−0·05461 ± 0·01102 (4·95)	2·44	−0·09227 ± 0·01083 (8·55)
Cutaneous	+0·04298 ± 0·01743 (2·46)	1·84	+0·08135 ± 0·01170 (6·95)

* The significance of the coefficients is indicated by the values (in brackets) of b/σ_b; the significance of the interspecific differences is indicated by values of $b_L - b_P/\sigma_{(b_L - b_P)}$.

Determination of pulmonary and cutaneous oxygen uptake

The apparatus described above, and illustrated in Fig. 1, was specifically designed to make possible the simultaneous but separate determination of oxygen uptake via the lung and via the skin under conditions as close to normal as practicable. It was used to investigate the relationship between cutaneous and pulmonary oxygen consumption over a wide range of dissolved oxygen tensions in animals which were allowed access to atmospheric air for lung-filling.

Because of the uncertainty about the end of the diving period in *Lymnaea* (see above) it was not possible to limit observation periods individually according to diving behaviour. Accordingly, a fixed period was adopted for each species; 30 min for *Lymnaea* and 60 min for *Planorbis*. Each observation therefore represents the rate of oxygen uptake during a standardized 'dive' following a normal lung-filling. The difference in the observation periods for the two species represents a compromise between the need to allow an adequate change in dissolved oxygen concentration as a basis for estimating cutaneous uptake and the desirability of imitating the natural dive. Early measurements in Leiden suggested that *Planorbis* dives were longer than those of *Lymnaea*. Although re-examination of diving duration in Sheffield indicated that this difference is not significant in aquaria, there are reasons for believing that a substantial difference does occur in nature (see p. 19).

The extent to which these standard periods agreed with the 'mean natural dive' may be judged by comparing the mean per cent O_2 values for initial and final pulmonary air obtained in these experiments with those obtained from

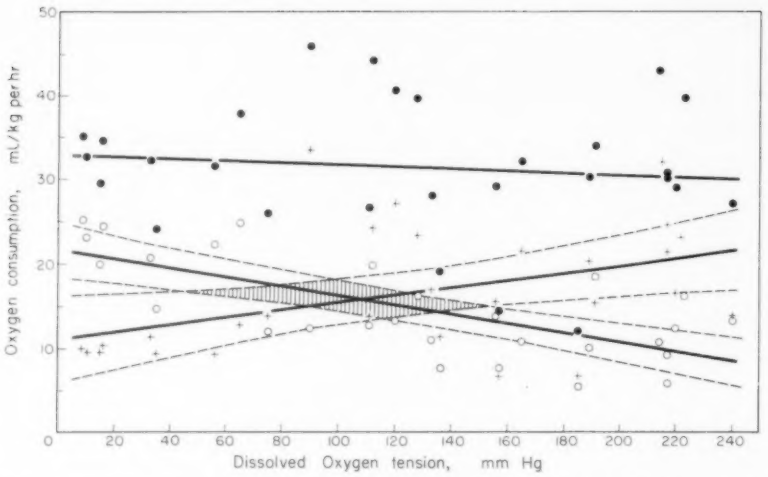

FIG. 3. Oxygen consumption of *Planorbis*—simultaneous determination of pulmonary (○), cutaneous (+) and total (●) uptake as a function of dissolved pO_2. Each trio of points represents a separate determination by the method described in the text; the values of pO_2 are the means of the determined tensions at beginning and end of the observation periods. For each set of points a regression line has been calculated (———) and for the cutaneous and pulmonary regressions confidence limits have been calculated (– – – – –).

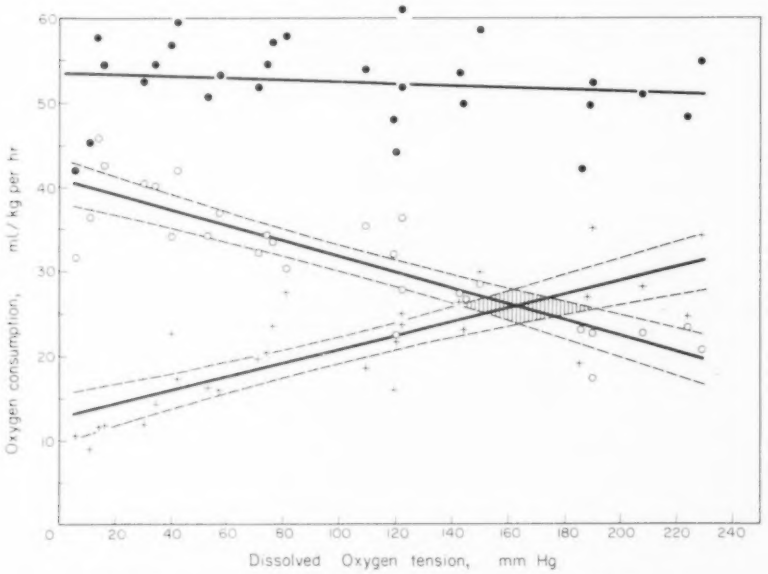

FIG. 4. Oxygen consumption of *Lymnaea*—indications as in Fig. 3.

animals diving in ditches and in aquaria (see Table 2). There is a reasonable correspondence in the initial per cent O_2 values for both species, though in each case the values found in the gas-exchange apparatus are a little lower than those found in nature. Final per cent O_2 values suggest that the standard 60 min period for *Planorbis* was satisfactory in imitating the mean time spent by the animal on the bottom (cf. aquarium values) though perhaps too short in relation to the overall period, including the journey to the surface, which is as a rule considerably longer under natural conditions (cf. ditch values). Final values for *Lymnaea* suggest that even the reduced standard period of 30 min was a little too long, though for the change of per cent O_2 in the lung there is good agreement between the ditch and apparatus figures.

The procedure for making the oxygen uptake measurements has been fully described under 'Methods'; the results are represented graphically in Figs. 3 and 4, while Table 4 gives the values of linear regression coefficients calculated for the various sets of data and fully discussed below. The data are derived from observations on ten specimens of *Planorbis* (wet wt. including shell 2·5–3·2 g) and eight specimens of *Lymnaea* (wet wt. 3·1–3·6 g), the oxygen consumption of each snail being measured in several parts of the pO_2 range. On several occasions *Planorbis* became completely inactive, withdrawing into the shell during part of the observation period; in such cases the observations were abandoned. In view of the difference in history of the representatives of the two species (see 'Materials'), six observations were made on three *Planorbis* which were brought into the standard laboratory conditions in June; they were used 3–4 weeks after the transfer. The data obtained with these animals conformed with the pattern obtained with animals which had over-wintered in the laboratory.

PHYSICAL CONDITIONS IN THE NATURAL HABITAT

A number of measurements were made in the ditch from which the experimental animals were collected. Unfortunately it was only possible to carry out such observations in summer. Dissolved oxygen concentrations and water temperatures were measured near the surface, among clumps of aquatic plants, and close to the bottom mud at various times of the day. The depth of water was about 15 cm. In addition a few measurements of pCO_2 were made by shaking a small bubble of air in a syringe containing the water sample. After equilibration the bubble was analysed in Krogh's micro-gas-analyser. These samples for pCO_2 were taken at and shortly after sunrise from the surface layers among clumps of aquatic plants which in June were filling the ditch. These samples were thought to give an indication of the maximum values likely to be encountered by the animals. The highest pCO_2 was approximately 15 mm.

Dissolved oxygen and water temperature data are presented in Fig. 5. On two hot sunny days in June surface oxygen measurements covered the remarkable range from 0·4 ml/l. at 3.30 a.m. (just before sunrise) with a temperature of 13°C to 16·9 ml/l. at 1.30 p.m. with a temperature of 30°C. These minimal and maximal values correspond to a range of pO_2 from about 10 to 490 mm. The

surface water was already supersaturated by 8.30 a.m. and was still well above saturation when the last sample was taken at 9.30 p.m. The pO_2 probably exceeded atmospheric tension for 14 or 15 h/day under these circumstances. Samples taken close to the bottom started from a value about twice that for the surface at the same time and reached a maximum of 4·3 ml/l. (≡ *ca.* 100 mm) about mid-day. These levels occurred at a time when the ditch was choked with plants so that circulation caused by wind must have been very restricted.

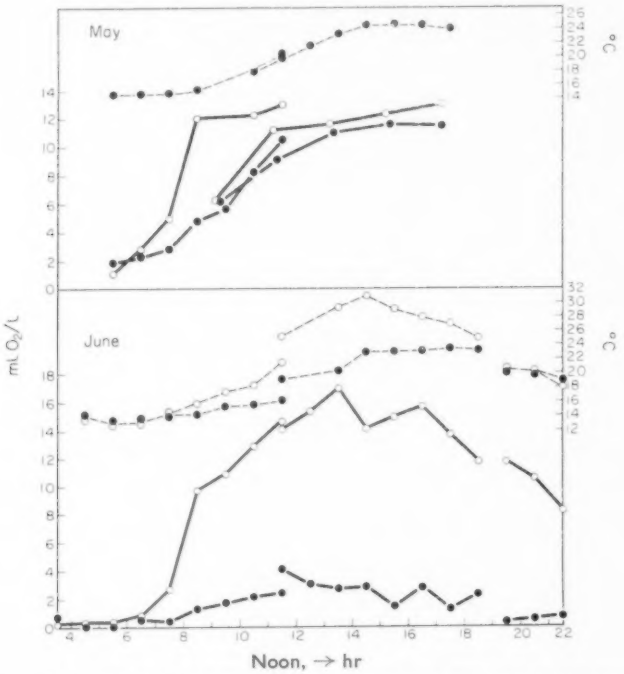

FIG. 5. Temperature and dissolved O_2 variations in the ditch from which the experimental animals were collected. Observations on two sunny days in May and two sunny days in June 1960. ○- - - - -○ surface and ●- - - - -● bottom temperatures (°C); ○——○ surface and ●——● bottom O_2 concentrations (ml/l.); depth of water about 15 cm.

In May when plant growth was much less abundant, the situation was rather different, presumably because of greater water circulation. The maximum concentration at the surface was about 13·1 ml/l. (≡ 340 mm) and at the bottom 11·4 ml/l. (≡ 300 mm). It is clear that when plant growth is abundant, animals near the surface will be exposed to exceptionally high oxygen tensions for large parts of each day. Conditions near the bottom will depend on circulation conditions, but may also reach a state of considerable oxygen supersaturation.

These findings confirm and in some respects extend those reported by Zaaijer and Wolvekamp (1958) whose observations refer to a similar ditch.

A NOTE ON THE PRESENCE OF HAEMOCYANIN IN *LYMNAEA*

Since it was hoped that a comparison of the respiratory capabilities of *Planorbis* and *Lymnaea* would throw some light on the function of haemoglobin in the former, it seemed desirable to confirm that no respiratory pigment is present in the blood of the latter. Füsser & Krüger (1951) made determinations of the copper content of *Lymnaea* haemolymph and found 0·0125 mg/ml, corresponding to about $\frac{1}{6}$th

TABLE 5—COPPER CONTENT AND OXYGEN CONTENT OF *Lymnaea* BLOOD*

Copper content (mg/ml)	Total O_2 (vols %)	Bound O_2 (a) (vols %)	Bound O_2 (b) (vols %)
0·0068	0·72	0·12	0·12
0·0068	0·72		
0·0021	0·74	0·12	0·04
0·0021	0·70		
0·0013	0·56	−0·04	0·02
0·0013	0·56		
0·0018	0·69	0·09	0·03
0·0018	0·68		
0·0148			

* Duplicate determinations were made on four separate samples each of which represented the pooled blood of some twenty animals. The final sample was obtained in a different manner as described in the text and was sufficient only for a single Cu determination. Two values for bound oxygen are derived (a) by subtraction of an arbitrary amount (0·6 vols per cent) for dissolved oxygen (column 3) and (b) by calculation from the Cu content assuming the whole of this to represent haemocyanin (column 4).

that in *Helix* blood. They assumed that this represented haemocyanin. This matter was re-examined for me by Mr. G. L. Spoek of the Zoological Laboratory, University of Leiden, who has kindly permitted me to include the following summary of his findings.

Lymnaea blood was collected by causing the animal to contract violently into the shell. The hæmorrhage appeared to occur in the lung and the expelled fluid was of a pale orange colour with a distinct opalescence. From one group of animals a very small additional quantity of fluid was extracted by lacerating the foot after the maximum yield had been obtained by the other method. This was noticeably darker in colour. Pooled freshly collected blood from twenty snails furnished sufficient material for duplicate determinations of copper content and of total oxygen content (after equilibration with atmospheric air) by means of the van Slyke method.

The results of these determinations are shown in the first two columns of Table 5. The third column shows the deduced value for the bound fraction of the total oxygen, assuming that the dissolved oxygen accounted for 0·6 vols per cent, while the fourth column contains corresponding theoretical values for bound oxygen, assuming all the copper to represent haemocyanin. The last

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value (copper content only, because insufficient material was available for van Slyke determination) refers to residual 'blood' obtained by lacerating the foot. Only in this one case was copper content as high as reported by Füsser & Krüger (1951). While in all other cases still significant copper contents were found, there is poor agreement between the values for bound oxygen deduced from total oxygen content and those calculated from the copper content. It must be remembered, however, that these values lie close to the limit of sensitivity of the van Slyke method.

The sample which contained 0·0068 mg/ml of copper was very carefully examined with a spectrophotometer after equilibration with oxygen, but this failed to reveal any characteristic pattern of absorption. The typical oxyhaemocyanin curve could be obtained with *Homarus* blood diluted ($\times 13$) with saline to an equivalent copper content. It is possible that a relatively high content of other proteins in the *Lymnaea* blood acted as a masking factor.

A qualitative test for the copper-containing enzyme polyphenol oxidase gave a positive result.

Although the blood of *Lymnaea* contains a small amount of copper, there is at present no conclusive evidence that this represents haemocyanin. Even if it does, the concentration is too low for any significant contribution to oxygen transport to result.

DISCUSSION OF RESULTS

It was expected on *a priori* grounds, and demonstrated by the gas exchange observations, that the importance of pulmonary O_2 uptake increased as the dissolved pO_2 decreased. In Leiden it was not possible to demonstrate a consequent relationship between duration of the dive and pO_2, probably because of the excessive irregularity of the intervals. However, a repetition of these observations with English snails did indicate a significant influence of dissolved pO_2 on the duration of the dive in both species (Table 1). At any one pO_2 there was no significant difference between the species. These observations were made on snails diving in 1 l. jars and there are reasons for thinking that in natural circumstances an interspecific difference does occur (see below).

Amongst the factors which tend to obscure the relationship mentioned above, are variation in the activity of individual animals and variation in the initial volume and oxygen content of the pulmonary gas. Activity variations were only too apparent whenever a small number of animals was observed intensively in these and other experiments. Apart from omitting data from animals which withdrew into their shells during observation periods, little could be done to reduce this source of variation. That there is a substantial variation in the oxygen content and volume of pulmonary gas is clear from the figures in Tables 2 and 3 respectively.

In so far as diving periods are directly influenced by the rate of utilization of pulmonary oxygen, there are two means by which the behaviour could be regulated. The animals may respond to the lowering of pO_2 in the lung and/or the

reduction of the pulmonary gas volume with consequent increase of specific gravity or loss of buoyancy of the shell and its contents. That pulmonary pO_2 of itself is not a critical factor determining diving behaviour is suggested by the following points: (a) there is a substantial variation in the final pulmonary oxygen concentration (Table 2) even for animals diving in aquaria, where the interval between the onset of the stimulus and reaching the surface was small and relatively constant compared with natural conditions; (b) in some of the pressure experiments *Planorbis* was able to start its dives quite normally after filling its lung from an atmosphere with only 75 mm pO_2.

The bearing of animals which had been away from the surface for long periods suggested that change in buoyancy of the shell and its contents might act as a regulating factor in diving. The results of the pressure experiments strongly support this idea. It must be realized, however, that the extent of reduction of pulmonary gas volume used in these experiments was substantially greater than that found in any of the aquarium dives represented in Table 3. That such a large volume reduction was necessary to produce a clear-cut response in the pressure experiments may have been due to the high levels of pulmonary pO_2 which must have been a feature of these very short dives. The possibility of hydrostatic regulation also gains some support from the apparently greater constancy of final, as compared with initial, pulmonary gas volumes in *Planorbis* (Table 3). That this is not apparent in the *Lymnaea* figures is probably due to the uncertainty of recognizing the end of the dive in this species, under the conditions of these observations (see above).

The extent of the volume change in the air bubble during the dive in the gas-exchange apparatus ranges from 4 to 16·8 per cent in *Planorbis* with a mean of 9·2 per cent. There are reasons, which are discussed below, for thinking that under natural conditions the volume change is substantially greater. Since many of the *Lymnaea* final bubble volumes are too low, conclusions about the volume change in this species are also uncertain. However, if *Planorbis* and *Lymnaea* dives of similar length are compared (Table 3), it will be seen that the per cent volume changes are greater in the latter. This is consistent with the higher oxygen consumption and greater dependence on the lung in *Lymnaea*, as shown by the gas-exchange observations.

The values for pulmonary CO_2 included in Table 2 make it clear that in general (but see below) this gas does not accumulate in the lung and accordingly cannot play any part in regulation of diving behaviour.

One may postulate a mechanism for regulation of the dive involving at least two factors, viz., change in pulmonary pO_2 and change in pulmonary gas volume. The latter could act through a simple proprioceptor system sensitive to loss of buoyancy of the shell complex. In view of the unwieldy nature of an unbuoyant shell, this aspect of the mechanism would have additional non-respiratory advantages. It should be noted in this connection that young *Planorbis* have been seen to furnish themselves with air bubbles before the shell height reaches 2 mm. The respiratory value of the air bubble is presumably much less in animals of

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this size compared with mature animals and the hydrostatic function may be primary.

There is clearly room for a much more detailed analysis of the relationship between changes in pulmonary pO_2 and gas volume and diving behaviour.

A number of points emerge clearly from the analyses of initial and final lung bubbles (Table 2). There is no significant change in pCO_2 in either species. The range corresponds to about 8–15 mm for dives in the ditch and in aquaria; somewhat higher values (up to 22 mm) were found in the gas-exchange apparatus. The lung could perhaps play a significant part in the elimination of CO_2 when environmental levels are as high as those in the lung. As already noted, 15 mm pCO_2 was found in one water sample from the vicinity of a dense mass of aquatic plants at sunrise; higher values than this are very unlikely and for most of the time pCO_2 would be very much lower.

The oxygen figures in Table 2 indicate that in both species pulmonary pO_2 is always substantially below the atmospheric level. Ventilation of the lung is clearly imperfect and presumably varies with the duration of lung-opening; the influence of this factor on initial pO_2 has not been studied. The drop in per cent O_2 is approximately the same for both species in aquaria, but both initial and final mean per cent O_2 are significantly higher for *Lymnaea*. In the ditch the drop is significantly greater for *Planorbis*; although there is no significant difference between the initial means for the two species, the difference between the final means is highly significant. The mean of 4·2 per cent for *Planorbis* includes one exceptionally high value of 11·2 per cent and if this is omitted the mean final per cent O_2 becomes 2·8 per cent compared with 8·8 per cent for *Lymnaea*.

At this point it is necessary to elaborate on one aspect of the general behaviour referred to in the earlier description. Observations of snails, both in the natural habitat and in a simulated ditch in the laboratory, made it clear that there is an important difference in the range of the two species. While *Lymnaea* normally remains browsing on the plants and accordingly within easy reach of the surface, *Planorbis* spends a substantial part of its life creeping and feeding on the bottom mud. The latter, therefore, often has to make a considerable journey, when stimulated to seek the surface, in order to find a plant which can act as a 'ladder'. For *Planorbis* the time elapsing between the onset of the stimulus and arrival at the surface will accordingly be longer and more variable than for *Lymnaea*. In small aquaria, on the other hand, both species will have short journeys to the surface, an unnaturally easy situation for *Planorbis*. This contrast would account for the similarity between ditch and aquarium values of final per cent O_2 for *Lymnaea* and the corresponding dissimilarity for *Planorbis* (Table 2). It would also account for the lack of a significant difference between the species in the duration of aquarium dives (Table 1). Finally, it would follow that the per cent volume change during *Planorbis* aquarium dives is smaller than in nature, perhaps by as much as 50 per cent.

The data for oxygen consumption as a function of dissolved oxygen tension show a wide range of variation, particularly in the case of *Planorbis* which (as

already noted) is very variable in its activity. Füsser & Krüger (1951) commented on this difficulty in their own study on these species. All the original data for cutaneous, pulmonary and total O_2 uptake are plotted in Figs. 3 and 4. Linear regressions have been calculated for each of the six sets of data and the coefficients are given in Table 4. The regression lines are drawn in on Figs. 3 and 4 together with the confidence limits in respect of the cutaneous and pulmonary lines.

These confidence limits indicate the range $\pm 2\sigma_{\hat{y}}$, where $\hat{y}$ denotes the oxygen consumption estimate given by the regression line and $\sigma_{\hat{y}}$ is the standard deviation of the observed values about this line. The best estimate of $\sigma_{\hat{y}}$ for any given value x_0 of pO_2 is calculated from the formula

$$\sigma_{\hat{y}}^2 = \frac{1}{n-2} \cdot \left(B - \frac{C^2}{A}\right) \cdot \left(\frac{1}{n} + \frac{(x_0 - \bar{x}^2)}{A}\right)$$

where $A = \Sigma x^2 - (\Sigma x)^2/n$; $B = \Sigma y^2 - (\Sigma y)^2/n$ and $C = \Sigma xy - \Sigma x \,.\, \Sigma y/n$

The population mean for any particular value of x will lie within these limits with a probability of approximately 0·95. Regressions of cutaneous and pulmonary O_2 uptake were also calculated on the basis of a semi-logarithmic transformation (log Q_{O_2}/pO_2) and these were found to give a slightly better fit to the observed values than those calculated for arithmetic co-ordinates, especially at very low values of pO_2. This is not surprising since the cutaneous curves presumably should pass through the origin. However, the difference was too small to influence the conclusions set out below and in order to simplify the calculations based on the regression coefficients, the arithmetic co-ordinate basis was adopted.

Neither of the regressions of total O_2 consumption is significant but all the other regressions are significant at least at the 5 per cent level (see Table 4). As between species the regressions of cutaneous uptake are not quite significantly different but the difference between the regressions of pulmonary uptake is well above the 5 per cent significance level. The angle between the cutaneous and pulmonary regression lines is thus greater in the case of *Lymnaea* than in *Planorbis*. Accordingly, the following generalizations are warranted: (a) total O_2 uptake in each species is independent of dissolved pO_2; (b) cutaneous and pulmonary fractions are complementary—as dissolved pO_2 and cutaneous uptake fall, there is a compensatory increase in pulmonary uptake; (c) there is an obvious difference between the species in respect of the range of dissolved pO_2 in which pulmonary and cutaneous uptake are of equal importance (cf. stippled areas in Figs. 3 and 4)—thus pulmonary uptake exceeds cutaneous at dissolved pO_2 below 140 mm in *Lymnaea* but only below 40 mm in *Planorbis*, while cutaneous uptake exceeds pulmonary above 150 mm in *Planorbis* but only above 190 mm in *Lymnaea*; (d) values for the percentage of total uptake which is pulmonary are higher for *Lymnaea* than for *Planorbis* at all values of dissolved pO_2. The last two points clearly indicate that the lung is relatively more important in *Lymnaea* than in *Planorbis*. This difference may be attributed to the lack of an accessory gill and the presence of a consistently higher pulmonary pO_2 in the former species. It is interesting to note that the mean total oxygen consumption of *Planorbis* amounts

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to only 60 per cent of that of *Lymnaea*, a figure which agrees precisely with the findings of Füsser & Krüger (1951), in spite of the fact that they measured the oxygen consumption of their animals in moist air.

GENERAL DISCUSSION

All the studies of the oxygen-binding properties of *Planorbis* haemoglobin, referred to in the introduction, indicate a high oxygen affinity. The question has to be answered, whether, under natural conditions, the animal is exposed to oxygen tensions low enough to result in appreciable dissociation of the pigment when the blood reaches the tissues. Ideally one would determine the pO_2 and pCO_2 of arterial and venous blood under appropriate external conditions and refer these values to the appropriate dissociation curve. Since this is impracticable in the case of an animal as small as *Planorbis*, one may attempt some assessment of the state of saturation of the pigment *in vivo* while varying the external pO_2.

Some observations of this kind have been made by Leitch (1916) and by Fox (1945), the state of the haemoglobin in the extended foot being examined spectroscopically in each case. Leitch found that down to 7·7 per cent (*ca.* 60 mm) external pO_2 the blood was always saturated, while at 7·2 per cent (55 mm) "the blood is reduced". Fox, on the other hand, found that the external pO_2 corresponding to the complete disappearance of the oxyhaemoglobin absorption bands was 25 mm at 17°C; in some individuals the bands disappeared at a somewhat higher (undetermined) tension. A difference of 30–35 mm between the external tensions corresponding to full oxygenation and full deoxygenation appears excessive in the light of the dissociation curves constructed by Zaaijer & Wolvekamp (1958). These authors have discussed some factors which might account for the apparent discrepancy. They consider that a temperature difference could be only partly responsible (Leitch did not report the temperature), but two other factors might contribute. In the first place, there might have been a difference in the pO_2 in the lung, which was not taken into account in either observation. Secondly, stagnation of the water close to the animal could give an erroneous value for the critical external tension. In addition, it should be realized that it is uncertain whether the observed condition of the haemoglobin in the foot represents venous or arterial conditions. A drop of 35 mm in external pO_2 would not necessarily result in a similar fall in venous pO_2; in fact the buffering action of a respiratory pigment would be expected to lessen this effect.

It is evident that there is considerable uncertainty about the diffusion barrier at the respiratory surface. It must be remembered, moreover, that there are two or even three respiratory surfaces, the general exposed cutaneous surface, the accessory gill and the mantle epithelium (lung). At each of these sites critical tensions will be related to a complex of factors including diffusion distances, extent of vascularization and relative surface areas. It is worth noting that in *Planorbis* and *Lymnaea* some 32 and 41 per cent respectively of the total oxygen uptake is via the lung (where pO_2 is always considerably less than 158 mm) when dissolved pO_2 is 220 mm. On the other hand, a measurable part of the uptake

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still occurs through the skin or gill when dissolved pO_2 is as low as 15 mm. A further point of importance in discussing the relative importance of the various sites of oxygen uptake is their respective relationships to the general circulation. Whereas the lung receives all blood returning to the heart the accessory gill and the lacunae of the foot receive only part of the blood which is on its way to the heart (Zaaijer & Wolvekamp, 1958).

Since under natural conditions, *Planorbis* will always have access to atmospheric air, it is useful to establish whether in normal dives the pulmonary pO_2 falls below the critical level. That is the level below which the corresponding blood pO_2 will be inadequate to supply the demand for oxygen without falling below the loading tension of the pigment. This level is not likely to be higher than the 7·7 per cent found by Leitch. The data given in Fig. 2 show clearly that mean final pulmonary pO_2 falls well below this level during extended dives in the ditch but not during the 'easy' aquarium dives. It seems clear, therefore, that the pigment will function in oxygen transport during the latter part of the natural dive. This will be true whatever the dissolved pO_2, if the termination of the dive is governed in any way by the utilization of pulmonary oxygen. At high levels of dissolved pO_2 the dives will simply be longer and the critical pulmonary level will be reached more slowly. The present work does not support the statements of some authors (Leitch, 1916; Wesenberg-Lund, 1939) that *Planorbis* does not refill the lung in well-aerated water. In water of low pO_2 the pigment will probably contribute also to transport of oxygen absorbed through the skin and gill.

By contrast, the mean final pulmonary pO_2 in *Lymnaea* does not fall to 7·7 per cent even in natural dives. This means that if diffusion conditions are similar in this species and if the dissolved oxygen transport is similarly adequate at higher blood tensions, a respiratory pigment would serve no useful function in terms of the natural conditions of pulmonary oxygen uptake. In considering whether *Lymnaea* is at any disadvantage without a pigment so far as cutaneous uptake is concerned one can only point to two mitigating circumstances: (a) the somewhat superior pO_2 of the surface waters in which *Lymnaea* tends to remain; (b) the greater dependence on the lung compared with *Planorbis*.

In conclusion the following hypothesis is advanced concerning the function of haemoglobin in *Planorbis* and the success of *Lymnaea* without a pigment. In a habitat which is characterized by violent diurnal fluctuations of dissolved pO_2, both species are heavily dependent on the uptake of oxygen from the lung. Since *Lymnaea* depends entirely upon oxygen transported in solution in the blood, its penetration below the surface is limited, both as regards time and distance, by the necessity to maintain the pulmonary pO_2 at an adequate level. This restriction to regions within easy reach of the surface is related to its habit of browsing on submerged vegetation where, during the hours of daylight, the dissolved pO_2 is invariably high. *Planorbis* by virtue of its high oxygen affinity haemoglobin is able to exploit its pulmonary oxygen supply to a considerably greater extent and is therefore free to make dives of longer duration. This in turn enables it to

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journey further from the surface and spend much of its time browsing on the bottom mud. Possession of the pigment fits it for the occupation of a different ecological niche. Both species will at times be subject to very low dissolved pO_2, though under certain conditions this hazard will be more pronounced on the bottom. The role of *Planorbis* haemoglobin is not primarily related to survival in water of low pO_2, though in this respect it probably helps, but to longer survival away from the surface and atmospheric air.

SUMMARY

1. *Planorbis corneus* and *Lymnaea stagnalis* were compared in respect of a number of aspects of respiratory behaviour and gas-exchange. The gas-exchange measurements were made by an original method, which is fully described, enabling pulmonary and cutaneous uptake of oxygen to be determined separately but simultaneously.

2. In both species dives are longer when dissolved pO_2 is higher but in small aquaria there is no interspecific difference.

3. Both species respond to pressure increase in a manner which suggests that the pulmonary air may have an additional hydrostatic function and that buoyancy may play a part in regulation of diving.

4. Change in pulmonary per cent O_2 during the dive is similar in ditch and aquarium for *Lymnaea* but the change is much greater in the natural habitat in the case of *Planorbis*. There are reasons for thinking that *Planorbis* natural dives are longer than those in aquaria and observed differences in natural range between the two species are discussed.

5. Mean final per cent O_2 in the lung is much lower for *Planorbis* (2·8 per cent) than for *Lymnaea* (8·8 per cent).

6. The total oxygen consumption of *Planorbis* amounts to 60 per cent of that of *Lymnaea*. In both species cutaneous uptake increases as dissolved pO_2 falls and there is a compensatory increase in pulmonary uptake, so that total uptake is maintained approximately constant over the range 15–220 mm dissolved pO_2. The lung appears to be slightly more important in *Lymnaea* than in *Planorbis*, but in either case it still accounts for 40–30 per cent of the total uptake when conditions for cutaneous uptake are most favourable.

7. Observations on dissolved pO_2 in the natural habitat indicate that in summer, at least, there is a violent diurnal fluctuation (due to the activity of aquatic plants); pO_2 may range from 10–490 mm in 10 h.

8. A small amount of copper was found in the blood of *Lymnaea*. It was not possible to establish positively that this represents haemocyanin.

9. The new data are discussed in relation to the known properties of *Planorbis* haemoglobin. The hypothesis is advanced that the primary function of this pigment is to facilitate greater exploitation of the pulmonary O_2 store during each dive, rather than the uptake of O_2 from water at low tension. This permits

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longer dives and penetration to the bottom of the ditch. *Lymnaea* lacking a respiratory pigment maintains its pulmonary pO_2 at a higher level by shorter dives and so occupies a separate ecological niche nearer the surface.

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PHYSIOLOGY OF MOLLUSCA—I. ACTION OF ACETYLCHOLINE ON THE HEART OF *STROPHOCHEILOS OBLONGUS*

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Abstract—1. The action of drugs on the perfused isolated ventricle of the snail *Strophocheilos oblongus* is described.

2. ACh has an excitatory action on the heart.

3. An antagonism between ACh-atropine was found in the heart.

4. The cardio-accelerator present in *Strophocheilos* heart tissue extract is not ACh.

INTRODUCTION

THE present paper will describe the action of acetylcholine, and certain drugs known to be acetylcholine antagonists or potentiators, on the isolated ventricle of a common South Brazilian snail, *Strophocheilos oblongus* (Gastropoda, Pulmonata).

METHODS

Each experiment involved the use of 6 to 10 hearts.

The heart was cannulated and its beat recorded on a smoked drum.

The following perfusion fluid was devised in order to maintain the heart beating regularly for 1 or 2 days: 5 g/l. NaCl; 0·08 g/l. KCl; 0·6 g/l. $CaCl_2$. Different concentrations of these salts were tried in the composition of the perfusion fluid. All available perfusing solutions recommended for Helix (Zetler & Schlosser, 1954; Ripplinger, 1957; Binet *et al.*, 1955; Jullien *et al.*, 1954) did not give a regular beat and the *Strophocheilos* heart slowed down and stopped within minutes. The heart proved to be quite sensitive to changes in K and Ca. A KCl concentration of 0·02 g/l. made the heart beat irregularly and with small amplitude. Concentrations higher than 0·1 g/l. decreased the amplitude as well as the frequency. $CaCl_2$ concentrations proved to be very critical for regular frequency and amplitude. The reaction to $CaCl_2$ in lower concentrations than 0·6 g/l. was a decrease in amplitude with maintaining of tonus. Higher concentrations decreased tonus and rate.

RESULTS

Acetylcholine (ACh)—(Roche). When acetylcholine is added to the perfusion fluid running through a fresh beating *Strophocheilos* heart, concentrations lower than 10^{-7} g/ml produce no regular reaction, though a small decrease in amplitude is sometimes observed. Dilutions of acetylcholine between 10^{-7} and 10^{-6} have a positive inotropic and chronotropic effect, the threshold being in the vicinity of 10^{-7}. At concentrations of 10^{-5} and higher the effect is also inotropic positive

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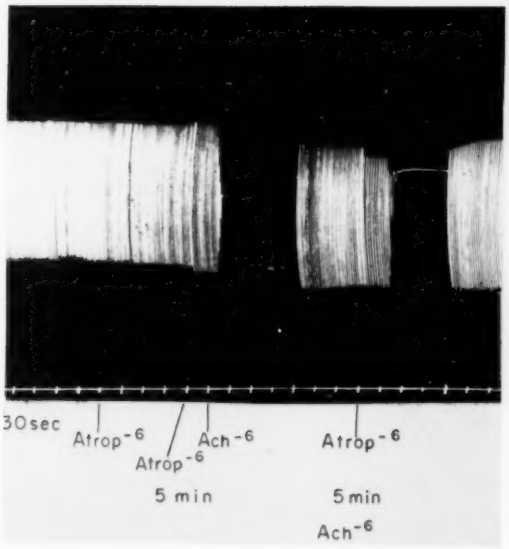

FIG. 1. The action of atropine and acetylcholine on the snail heart. Atropine by itself has no effect. If atropine is given and, 5 min later, acetylcholine added, the heart stops.

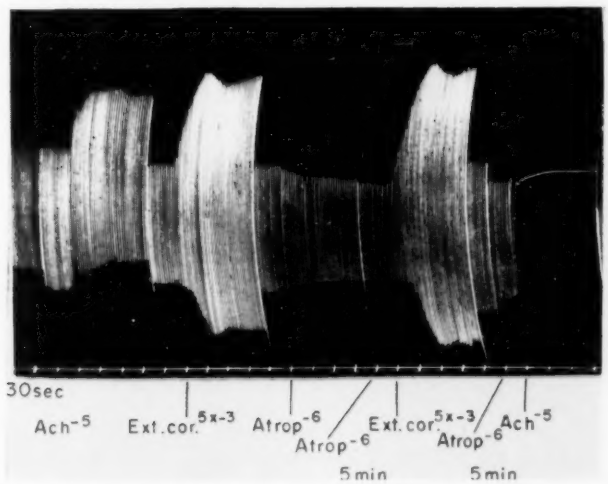

FIG. 2. Action of acetylcholine and heart extract (Ext. cor.). Acetylcholine causes an increase in the amplitude of the heart beat; so does the heart extract. Atropine will not have any effect by itself, neither does it inhibit the heart extract. It does inhibit the action of acetylcholine. Thus the heart extract is not effective due to any contained acetylcholine.

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but turns chronotropic negative. Acetylcholine at 10^{-6} was found to produce 32 to 66 per cent increase in amplitude of heart beat.

Eserine—(Merck). Acetylcholine was assayed on eserinized hearts. Concentrations of eserine at 10^{-6} to 10^{-4} were allowed to bathe the heart for 2–10 min prior to ACh treatment. Eserine failed to work as a potentiator of ACh on this heart nor did it show any other action.

Neostigmine—(Mann). The heart treated with the anticholinesterase neostigmine showed no reaction to the drug nor was the action of ACh significantly potentiated.

Mytolon. Mytolon chloride or 2.5-bis(3′-diethylaminopropylamine)-benzoquinone bis-benzyl chloride (Winthrop), a known active ACh blocking agent on the *Venus* heart (Ludueña & Brown, 1952), was tried on the *Strophocheilos* heart and we found that it excites the heart with an inotropic positive effect. Mytolon at 10^{-5} gives a revisible increase in amplitude of heart beat equal to a dose of ACh at 10^{-6}. Using ACh at 10^{-6}, when Mytolon has been in the bath for 15 min, a negative inotropic and chronotropic effect is seen. The threshold for Mytolon is around 10^{-6}.

Atropine—(Merck). When atropine was tested on this heart it produced a slight decrease in amplitude at a concentration of 10^{-6}, at least on some hearts. The constant reaction was to a later addition of ACh at 10^{-6} which promptly stops the heart in most cases or at least sharply decreases its amplitude (20–100 per cent). The action is easily reversed on washing so that the event can be repeated in the same heart (Fig. 1).

Histamine—(Pfanstiehl Chemical Co.). Histamine has a slight excitatory action on the *Strophocheilos* heart, the threshold being in the vicinity of 10^{-5}. It seems not to interfere with ACh action on the heart.

Nicotine—(Eastman Organic Chemicals). The action of nicotine on the *Strophocheilos* heart at a concentration of 10^{-5} proved to be most of the times inhibitory, giving a decrease in amplitude of 15–100 per cent. A slight antagonistic action to ACh at 10^{-6} was usually revealed when nicotine at 10^{-4} was left in the bath for 5 min.

Tissue extracts. Tissue extracts made of the *Strophocheilos* nervous ring and heart were assayed on the heart preparation. Fresh tissue after being weighed, and boiled for 5 min, was homogenized in a glass homogenizer with perfusion fluid. The final solution was always in terms of g of fresh tissue per ml of fluid. Both tissues have a powerful excitatory action on the heart and are not blocked by atropine. The positive inotropic effect of heart extracts at a concentration of 4×10^{-3} was matched by ACh at 10^{-5}. Pharmacological investigation on the nature of this cardio-accelerator present in the *Strophocheilos* tissue extracts are in progress in this laboratory (Fig. 2).

DISCUSSION

The isolated heart ventricle of the South American snail *Strophocheilos oblongus* used in this work proved to be a sensitive test organ for acetylcholine. It appears

also to be quite suitable for pharmacological assay of drugs and tissue extracts, since it is possible to maintain the ventricle beating for many hours with much regularity on a perfusing solution containing three salts. Acetylcholine produces positive inotropic and chronotropic effects on this heart beginning at 10^{-7} g/ml. Higher concentrations have an increased action until the heart is stopped in systole. The reaction of this heart to ACh is always excitation while a depressing reaction is reported for the *Helix pomatia* heart (Ripplinger, 1957). This is a peculiar difference for animals placed in so close a systematic position. On the other hand, the application of heart tissue extract on the perfused heart also has an excitatory power, the same happening for *Helix aspersa* (Kerkut & Laverack, 1960) and an opposite effect on the *Helix pomatia* heart (Ripplinger, 1957). Our results show that the cardio-accelerator factor present in the *Strophocheilos* heart tissue extract is not acetylcholine because an antagonism acetylcholine-atropine was found for the heart. An antagonism such as this failed to occur between extract-atropine. Mytolon, described by Welsh & Taub (1953) as one extremely effective acetylcholine antagonist on the *Venus* heart, does not work likewise on the *Strophocheilos* heart. Mytolon excites fresh hearts as does ACh, so a later addition of this ester is hidden by the gain in amplitude produced by Mytolon. Eserine, histamine and neostigmine failed to show any persistent measurable action in relation to ACh. The only inhibitory effect on the heart was produced by nicotine, which also failed to be a qualified ACh antagonist. On the other hand, atropine was the only effective ACh antagonist so far found for this heart.

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NUCLEOSIDE METABOLISM BY MONKEY BRAIN EXTRACTS*

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Abstract—1. Monkey brain extracts metabolize the ribose moiety from purine ribonucleosides to form a variety of phosphate esters which have been separated and identified by paper chromatography.

2. These products are formed by the successive action of nucleoside phosphorylase, transketolase and the glycolytic enzymes.

3. Phosphoglucomutase was isolated as partially purified fraction, and the presence of phosphoribomutase activity was indicated by the synthesis of inosine from xanthine and ribose-5-phosphate and by the accumulation of ribose-5-phosphate from nucleoside incubations.

4. The conversion of the purine moieties from nucleosides to free bases also has been investigated and the relationship of these changes to the rate of nucleoside metabolism is discussed.

INTRODUCTION

EXTENSIVE data have been documented on the enzymatic activities of mammalian brain preparation (Richter, 1957); however, few of these studies concern the sequential activities of a group of enzymes under conditions where intermediate products accumulate so that they can be identified and measured. Such a sequence of enzymatic reactions was noted and reported from a study of the metabolism of ribose derived from nucleosides, by rat, rabbit and guinea pig brain extracts (Rappoport *et al.*, 1960). By separating intermediate products, it was demonstrated that the ribose moiety is metabolized via the sequential operation of the hexose monophosphate shunt and the glycolytic pathways. A similar study was undertaken with monkey brain extracts in order to contrast the pentose metabolism by primate tissue with the results obtained with similar extracts from rodents.

Robins *et al.* (1953) studied nucleoside metabolism by monkey brain preparations and they indicated that phosphoribomutase was absent from this tissue. Since the conversion of nucleoside ribose to triose and hexose phosphates (Dische & Shiguera, 1957) requires the participation of a phosphoribomutase, or a phosphoglucomutase, it was necessary to re-examine ribonucleoside metabolism by monkey brain extracts to clarify this point.

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This report summarizes the findings on the nature of phosphate esters and purine derivatives formed from the metabolism of guanosine, adenosine and inosine by monkey brain extracts, and presents evidence for the occurrence of phosphoglucomutase in these extracts.

EXPERIMENTAL

Fresh or frozen monkey brains were homogenized in cold 0·1 M Tris$^+$ buffer (pH 7·4) in isotonic KCl, using 2 ml of homogenizing medium for each gram of tissue. The homogenates were centrifuged at $20{,}000 \times g$ for 15 min and the supernatant was separated from the insoluble pellet. The incubations were carried out in air at 37°C for varying time intervals. The incubation mixture contained 1·0 ml of brain extract, 5 μmole of nucleoside, 10 μmole of KH_2PO_4-P^{32} (pH 7·4), 1 μmole of $MgCl_2$ in 0·05 M Tris buffer (pH 7·4) to a total volume of 2·0 ml. The reaction was stopped with cold 25 per cent trichloroacetic acid and the acid-soluble fraction was separated by centrifugation.

Pentose was determined by the Mejbaum (1939) orcinol method, and the pentose moiety in nucleosides was distinguished from the pentose phosphates by destroying the latter with alkali before analysis (Ashwell, 1957). Deoxyribose was determined by a modified Dische cysteine procedure (Ashwell, 1957), sedoheptulose by the orcinol method described by Horecker *et al.* (1953), and lactic acid by the Barker & Summerson (1941) procedure. Sedoheptulose phosphate and ribulose phosphate were identified by an orcinol spray (Benvenue & Williams, 1951) after the phosphate esters were separated by paper chromatography and each ester located by radioautography on X-ray film by the procedure of Rappoport & Chen (1960). Purines and nucleosides were separated by paper chromatography (Benson *et al.*, 1950) and located by ultraviolet absorption. All spectral determinations were made using a Beckman DU ultraviolet spectrophotometer.

Frozen monkey brains, stored at -15°C for several weeks, were used for the isolation of phosphoglucomutase by the procedure described by Najjar (1955). This enzyme was tested by the conversion of glucose-1-phosphate to glucose-6-phosphate.

Experiments on the biosynthesis of inosine from hypoxanthine and ribose-5-phosphate were carried out with monkey brain extracts prepared as above and then dialyzed against 0·05 M Tris buffer (pH 7·4) for 18 hr at 5–7°C. Incubation mixtures containing 1·0 ml of dialyzed extract, 5 μmole of ribose-5-phosphate, 5 μmole of hypoxanthine, 1 μmole of $MgCl_2$ were made up to a volume of 2·0 ml with a final concentration of 0·05 M Tris buffer (pH 7·4). Controls constituted incubations with either hypoxanthine or ribose-5-phosphate. Incubations were carried out in air at 37°C for 2 h.

RESULTS

The extent of utilization of guanosine, adenosine, inosine and ribose-5-phosphate by monkey brain extracts is illustrated in Table 1. Ribose-5-phosphate and guanosine were utilized to a much greater extent than adenosine or inosine.

Ribose was not utilized under the same experimental conditions since no ATP was available (Sable, 1952). The rate at which nucleosides are metabolized is illustrated in Fig. 1 where the disappearance of guanosine and the simultaneous formation of pentose phosphates is shown. Metabolism of deoxyribonucleosides

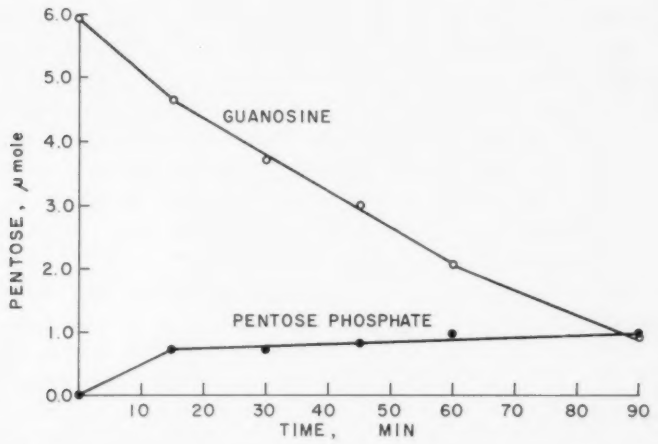

FIG. 1. A time study of guanosine metabolism and the formation of pentose phosphates by monkey brain extracts.

TABLE 1—NUCLEOSIDE AND PENTOSE METABOLISM BY MONKEY BRAIN EXTRACTS

Substrates*	Per cent metabolized
Guanosine	74
Adenosine	44
Inosine	42
Ribose-5-phosphate	82
Ribose	0
Deoxyguanosine	21
Deoxyadenosine	17
Deoxyinosine	15
Deoxyribose	0

* Incubation mixtures contained initially 5 μmole of each substrate. Incubated for 2 h under conditions described in the experimental section.

was studied and the results are shown in Table 1. All the deoxyribonucleosides tested were only slightly metabolized as compared to the corresponding ribonucleosides and no phosphate esters were detected in these incubations. Deoxyribose was not metabolized. No accumulation of lactic acid was noted in either ribo- or deoxyribonucleoside incubations.

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Radioautograms of the phosphate esters formed at different time intervals from guanosine metabolism by monkey brain extracts and separated by two-dimensional chromatography are shown in Fig. 2. The esters formed are glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate, ribulose-5-phosphate, sedoheptulose-7-phosphate, 3-phosphoglyceric acid, 2-phosphoglyceric acid and glycerol phosphate.

It is noteworthy from Fig. 2 that in the first 20 min 3-phosphoglyceric acid, fructose-1,6-diphosphate and an unknown polyphosphate appear in appreciable amounts. Traces of 2-phosphoglyceric acid plus glycerophosphate and of ribose-5-phosphate are visible; however, none of the hexose phosphates or sedoheptulose phosphate have been formed. In 40 min the unknown polyphosphate practically disappears; now hexose phosphates plus sedoheptulose phosphate and a trace of ribulose-5-phosphate become visible and fructose-1,6-diphosphate and 3-phosphoglyceric acid increase in concentration. The concentration of ribose-5-phosphate and 2-phosphoglyceric acid plus glycerol phosphate are maximal at this time. At 60 min the concentration of ribose-5-phosphate decreases while ribulose-5-phosphate and hexose phosphates plus sedoheptulose phosphate increase; fructose-diphosphate and 3-phosphoglyceric acid remain at approximately the same concentration as in the previous chromatogram, while 2-phosphoglyceric acid plus glycerol phosphate show some decrease in concentration. After 100 min, the concentration of fructose diphosphate, 3-phosphoglyceric acid, ribulose phosphate and hexose phosphates plus sedoheptulose phosphate remain high, while 2-phosphoglyceric acid and ribose-5-phosphate show further decrease in concentration. A trace of DPG is visible only in the chromatograms from the 100 min incubation mixture. Similar products were obtained when adenosine or inosine was used as a substrate.

Since the formation of sedoheptulose and hexose phosphates was noted (Fig. 2), the presence of an active phosphoribomutase or phosphoglucomutase for the conversion of ribose-1-phosphate to ribose-5-phosphate was suspected. Experiments were designed to check the presence or absence of these enzymes. With dialyzed monkey brain extracts, incubations were made with hypoxanthine and ribose-5-phosphate as precursors for the synthesis of inosine. After 2 h incubation, the fraction soluble in trichloracetic acid was separated, the trichloracetic acid removed with ether, the aqueous phase concentrated under vacuum, and the purine derivatives separated by paper chromatography (Benson *et al.*, 1950). Figure 3 illustrates the formation of inosine (R_f 0·38) from hypoxanthine (R_f 0·51) and ribose-5-phosphate. Orcinol analysis on the eluted inosine spot confirmed the presence of ribose, and ultraviolet spectral data established the identity of the spot as inosine (Beaven *et al.*, 1955). The controls with either hypoxanthine or with ribose-5-phosphate did not show any inosine formation.

Phosphoribomutase and phosphoglucomutase activities have been shown to reside within the same fraction (Klenow, 1953), and since ribose-1-phosphate was not readily available as a substrate, isolation of phosphoglucomutase was attempted from the monkey brain extracts. By use of the procedure described by Najjar

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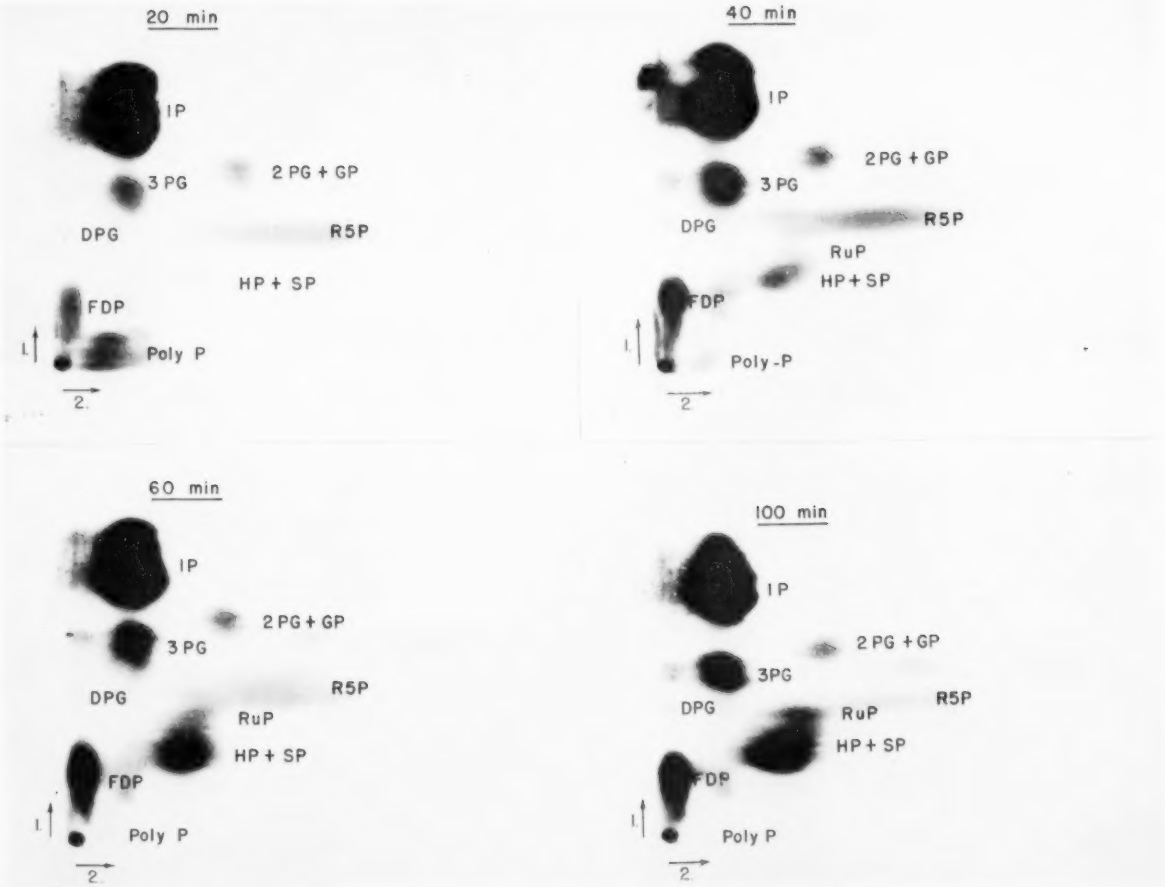

FIG. 2. Radioautograms of two-dimensional paper chromatograms of phosphate esters formed from guanosine during a time study. ATP, adenosine triphosphate; R5P, ribose 5-P; RuP, ribulose-P; Ga-3-P, glyceraldehyde-P; SP, sedoheptulose-P; GP, glycerol-P; DHA-P, dihydroxyacetone-P; G-1-P, G-6-P, glucose phosphates; F-6-P,-FDP, fructose phosphates; 2PG, 3PG, DPG, phosphoglyceric acids; HP, hexosephosphates (mixture of G-6-P + F-6-P).

(1)
Incubation
Mixture

(2)
Incubation
Mixture

Mixed
Standard

FIG. 3. Inosine (I), synthesized by monkey brain extracts from hypoxanthine and ribose-5-phosphate, separated from the incubation mixture by paper chromatography (13). This picture was made by direct contact of photographic paper with the chromatogram illuminated from below by an ultraviolet light source (256 mμ).

(1955), a tenfold purification of the phosphoglucomutase was achieved. The results from these isolation experiments are shown in Table 2.

Tests were made to determine what purine deaminases were present in monkey brain extracts. Incubations with and without inorganic orthophosphate in presence of guanine, guanosine, adenine and adenosine, respectively, were carried out and

TABLE 2—PHOSPHOGLUCOMUTASE IN CRUDE MONKEY BRAIN EXTRACTS AND PARTIALLY PURIFIED FRACTIONS*

Expt.	Fraction	Total Protein (mg)	μmoles G–6–P formed per mg protein per min
I	Crude extract	9870	0·29
	Fraction from 0·5–0·6 $(NH_4)_2SO_4$	63·0	2·60
	Fraction from 0·60–0·65 $(NH_4)_2SO_4$	43·0	2·34
II	Crude extract	2230	0·33
	Fraction from 0·5–0·6 $(NH_4)_2SO_4$	—	3·44

* Fractionation according to the Najjar (1955) procedure.

the products were examined spectrophotometrically and by means of paper chromatography (Benson *et al.*, 1950; Beaven *et al.*, 1955). If a guanosine deaminase was present in monkey brain, then, in absence of phosphate ion (without nucleoside phosphorylase activity), xanthosine should form from guanosine. Similarly with adenosine, inosine should accumulate. If the deaminase present in brain was active only with guanine or adenine, respectively, then accumulation of xanthine or hypoxanthine would be noted in those incubations in which the free purines were present alone as well as in those in which phosphate ions were present. Table 3 illustrates the results obtained. The monkey brain extracts did not deaminate guanosine or adenine, but an active guanine deaminase (guanase) and an adenosine deaminase were found in the extract. Fig. 5 illustrates the spectra of the substrates and the products formed from the bases and the nucleosides tested, and the findings are summarized in Table 3.

DISCUSSION

Monkey brain extracts metabolize purine ribonucleoside to form pentose phosphate, and sedoheptulose phosphate (Fig. 2) through the successive activities of nucleoside phosphorylase, phosphoribomutase, phosphoriboisomerase and transketolase (Kalckar, 1947; Dickens, 1958). The transketolase enzyme also catalyzes the transformation of pentose phosphates to triose phosphate and fructose-6-phosphate (Dickens, 1958), thus linking the above reactions with the glycolytic enzymes which yield fructose-1,6-diphosphate, glucose-6-phosphate, 3-phosphoglyceric and 2-phosphoglyceric acids (Fig. 2). Brain extracts from rat, rabbit and

guinea pig also form the same phosphate esters from purine riboside metabolism (Rappoport *et al.*, 1960).

The formation of the phosphate esters, described above and illustrated schematically in Fig. 4, requires the participation of phosphoribomutase or phosphoglucomutase since these two activities are believed to be the function of one

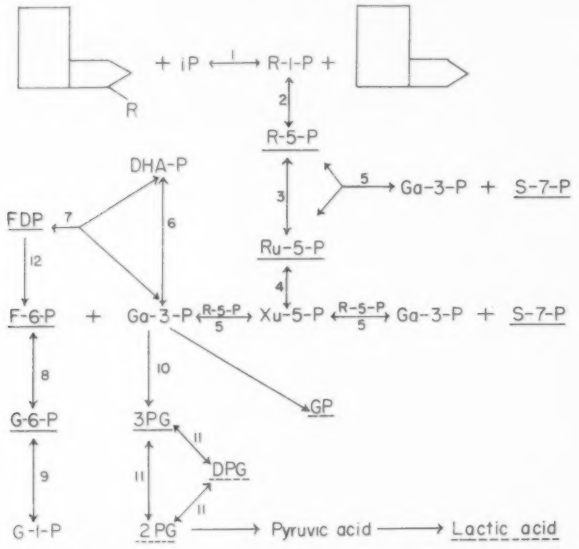

FIG. 4. Formation of phosphate esters from purine ribosides. Schematic representation of the enzymatic reactions involved in the formation of phosphate esters from guanosine by monkey brain extracts. Underlined solid lines indicate esters that have been identified; broken single lines indicate esters present are only in trace amounts. Enzymes indicated by number are as follows: (1) nucleoside phosphorylase, (2) phosphoribomutase, (3) phosphoriboisomerase, (4) phosphoketopentoisomerase, (5) transketolase, (6) triosphosphate isomerase, (7) aldolase, (8) phosphoglucoisomerase, (9) phosphoglucomutase, (10) triophosphate dehydrogenase, (11) phosphoglyceric mutase, and (12) fructose diphosphophosphatase.

enzyme (Klenow, 1953). The presence of phosphoglucomutase in monkey brain extracts is shown in Table 2. Formation of inosine from hypoxanthine and ribose-5-phosphate (Fig. 3) shows that phosphoribomutase is present in monkey brain extracts (reaction 1 below), and the reactions responsible for the synthesis are represented as follows:

1. Ribose-5-phosphate $\rightleftharpoons$ ribose-1-phosphate;
2. Ribose-1-phosphate + hypoxanthine $\rightleftharpoons$ inosine + phosphate.

The reactions involved in the conversion of ribose moiety from purine ribosides to a variety of phosphate esters are schematically represented in Fig. 4. This sequence of reactions accounts for all of the phosphate esters identified in this study of nucleoside metabolism by monkey brain extracts.

Examination of the changes in the purine moieties of guanosine and adenosine, respectively, during incubation with monkey brain extracts showed that a guanase and an adenosine deaminase were present (Table 3 and Fig. 5). Thus, the changes in the purine moiety during the metabolism of guanosine by these extracts can be summarized as:

1. Guanosine + phosphate $\rightleftharpoons$ guanine + ribose-1-phosphate;
2. Guanine + $H_2O \rightarrow$ xanthine + $2NH_4^+$;

where (1) is mediated by a nucleoside phosphorylase and (2) by guanase.

TABLE 3—DEAMINATION OF PURINE BASES AND NUCLEOSIDES BY DIALYZED MONKEY BRAIN EXTRACTS*

Substrate	Inorganic Phosphate	Products†
Guanosine	present	Xanthine
,,	absent	Guanosine
Guanine	—	Xanthine
Adenosine	present	Inosine + Hypoxanthine
,,	absent	Inosine
Adenine	—	Adenine

* Extracts were dialyzed against 0·05 M Tris buffer (pH 7·4) for 18 h at 5°C.

† Purine products were separated by paper chromatography (Benson *et al.*, 1950), eluted from the paper and identified by their ultraviolet spectra. Nucleosides were also tested for a positive orcinol reaction.

With adenosine as a substrate, the presence of an adenosine deaminase (Table 2, Fig. 5) and the utilization of inosine indicate the following reaction sequence:

1. Adenosine + $H_2O \rightarrow$ inosine + NH_4^+;
2. Inosine + phosphate $\rightleftharpoons$ hypoxanthine + ribose-1-phosphate.

Reaction (1) is catalyzed by a deaminase and (2) by nucleoside phosphorylase.

The accumulation of xanthine and hypoxanthine from guanosine and adenosine metabolism, respectively, by monkey brain extracts indicates that xanthine oxidase is absent in these preparations, similar to such observations in brain preparations from the rat, rabbit and guinea pig (Rappoport *et al.*, 1960). Thus, free bases formed from nucleic acids and nucleotide coenzymes can be utilized for their resynthesis as a cellular "salvage" process (Kornberg, 1957).

The dismutation of ribose from purine ribonucleosides to a variety of phosphate esters (Fig. 2), via the sequence of reversible actions indicated in Fig. 4, illustrates in detail the mode of interconversion of pentose phosphate to triose, hexose and sedoheptulose phosphates, thus relating the latter to the overall "turnover" of nucleic acids and nucleotide coenzymes within the cells. Knowledge as to the products formed in the catabolism of the pentose moiety also indicates the manner whereby the carbons from the pentose can be converted to CO_2 via pyruvate by mitochondrial oxidation, or to amino or fatty acids (Holzer, 1959). However, it is impossible from these studies to assess the contribution of the intermediates formed from the pentose moiety of nucleosides to the general economy of the cell.

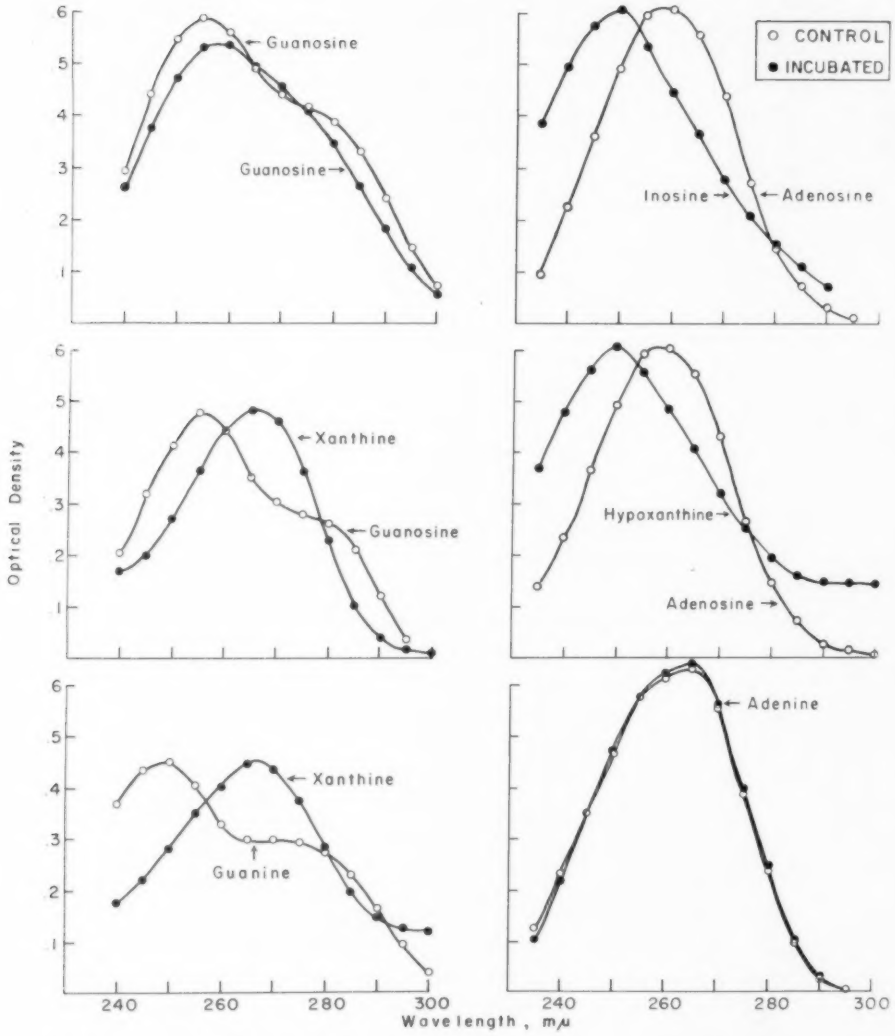

FIG. 5. Changes in ultraviolet absorption spectra when purines and purine ribonucleosides, respectively, were incubated with dialyzed monkey brain extracts with and without inorganic phosphate: top row and bottom row, in the absence of phosphate; middle row, in the presence of phosphate. Controls were incubation mixtures inactivated at the start of the experiment.

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THE EFFECT OF SODIUM IONS ON THE RESTING AND ACTION POTENTIALS OF LOCUST AND COCKROACH MUSCLE FIBRES

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Abstract—1. The resting and action potentials of skeletal muscle fibres of *Periplaneta americana* L. and *Locusta migratoria migratorioides* R. & F. have been measured in salines of varying sodium concentration.

2. As sodium is removed from the bathing medium, the resting and action potentials decrease in magnitude, and the time course of the rising and decay phases of the action potential is lengthened.

3. It is suggested that sodium may exert a general effect on the permeability to ions of insect muscle fibre membranes.

I. INTRODUCTION

THE presence of sodium ions in the external medium is necessary for the complete development of the end-plate potential (Fatt & Katz, 1952) and for the production of a propagated response (see Hodgkin, 1951) in vertebrate muscle fibres. In muscle fibres of the stick insect *Carausius morosus* Br., the removal of sodium ions from the bathing fluid results in an action potential of reduced size, but an active membrane response persists in addition to the junctional potential (Wood, 1957). Hoyle (1953) reported that the action potential of locust retractor unguis muscle fibres decreases in magnitude when sodium ions are removed from the bathing medium, but gave no details of this effect. No other studies of the action of sodium ions on the responses of insect skeletal muscle fibres have been made. The present paper is an account of such studies, which have been carried out on muscle fibres of the locust and cockroach.

II. METHODS

The insects used in this work were *Locusta migratoria migratorioides* R. & F. and *Periplaneta americana* L. The locust preparation was essentially that described by Hoyle (1955b). Both the flexor and extensor tibialis muscles of the metathoracic leg were investigated. In experiments on the cockroach, the animal was fastened to a bed of modelling clay and the required leg was surrounded by a wall of the same material. The test salines could be pipetted into and out of the well so formed. The muscles examined were the flexor tibialis muscle and posterior coxal depressor muscle no. 178 of Carbonell (1947) of the metathoracic leg.

In both species the cuticle was carefully removed from above the muscle to be investigated, which was then bathed in the test saline. The muscles were stimulated by the application of square pulses to their nerves in the thorax. Resting and action potentials were recorded by means of glass capillary intracellular micro-electrodes. The recording apparatus and sampling procedure were the same as those used previously by Wood (1957) in work on *Carausius*.

For locust muscles, the basic saline had the same composition as that used by Hoyle (1955a). The basic saline for cockroach muscles was based on analyses of cockroach blood, made for the purpose of this work, and using the techniques previously employed in analysing the blood of the stick insect (Wood, 1957). This saline had the following composition (mM/l.): Na, 100; K, 14; Ca, 4; Mg, 10; Cl, 142; HCO_3, 4; H_2PO_4, 6. The sodium concentration of the salines was varied by altering the NaCl content. For zero sodium salines, $NaHCO_3$ and NaH_2PO_4 were replaced by the corresponding potassium salts. In early experiments, tonicity was maintained by addition of the necessary quantity of sucrose. This was later replaced by choline chloride. All figures given in this paper refer to experiments in which choline-substituted salines were used, but it must be stressed that these results were not significantly different from those obtained in sucrose-substituted salines.

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III. RESULTS

The results obtained from all four muscles were identical, within the limits of experimental error. When the external sodium concentration was progressively lowered, there was a corresponding decrease in the magnitudes of the resting and action potentials (Fig. 1). Although the two curves in Fig. 1 have a similar general shape they are not parallel, particularly at the lower sodium concentrations. In locust muscle, del Castillo, Hoyle & Machne (1953) found that the size of the junctional potential (*j.p.*) was proportional to that of the resting potential. This was confirmed during the course of the present work and was also found to apply to the cockroach. The same relationship is present in stick insect muscles (Wood, unpublished observations). If the curve for the action potential deviates from that for the resting potential, the deviation should therefore be due to the active membrane response (*a.m.r.*). In some of the action potentials it was possible to distinguish between the *j.p.* and the *a.m.r.*, using the criterion previously employed by Hoyle (1955a) and Wood (1957). The curves for these two components are shown in Fig. 2. As expected, the curve relating *j.p.* to external sodium concentration is very similar in shape to that for the resting potential in Fig. 1, but the curve for the *a.m.r.* (Fig. 2) declines much more rapidly in low sodium concentrations than the curves for the resting potential and *j.p.* Extrapolation of the curve for the *a.m.r.* (Fig. 2) suggests that there is a critical level of sodium ions in the external medium of about 20 mM/l., below which an *a.m.r.* is not normally produced. However, in both the locust and cockroach, in several experiments using zero sodium salines, a plateau occurred at the peak of the action potential, or even a slight hump (Fig. 3(b)). In most cases in which this

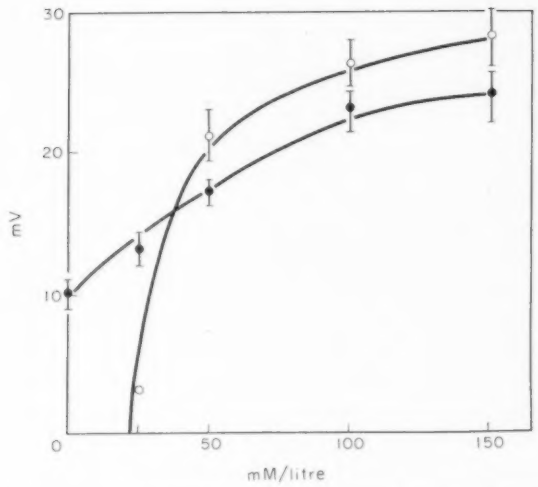

FIG. 1. Relation between external sodium concentration and size of resting potential ●, and action potential ○, in locust extensor tibialis fibres. Horizontal bars denote limits of S.E. of mean.

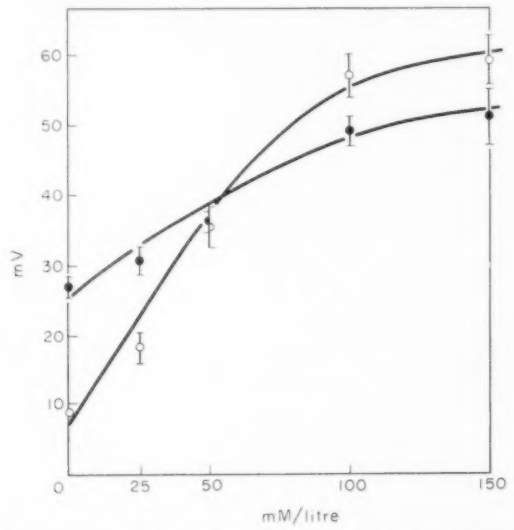

FIG. 2. Relation between external sodium concentration and size of junctional potential ●, and active membrane response ○, in cockroach flexor tibialis fibres.

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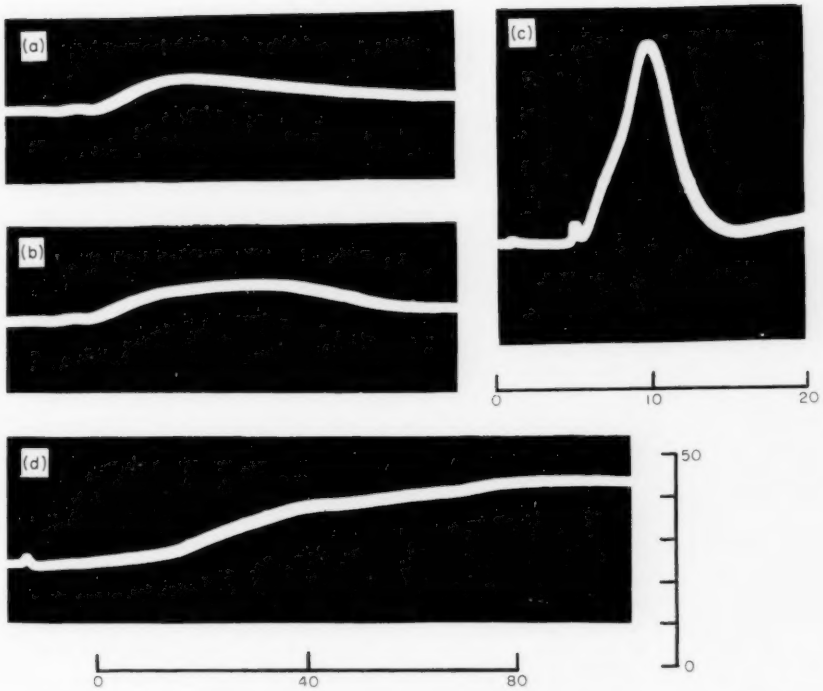

FIG. 3. Locust flexor tibialis muscle. a–c, records in zero sodium saline (upper time base). a, normal response. b, the same fibre stimulated again about 1 sec later. c, the same fibre 15 min after return to 150 mM sodium, showing tendency for initiation of *a.m.r.* to be delayed. d, record from another preparation on slower time base, showing an irregular and much lengthened response in zero sodium. Calibration: msec and mV.

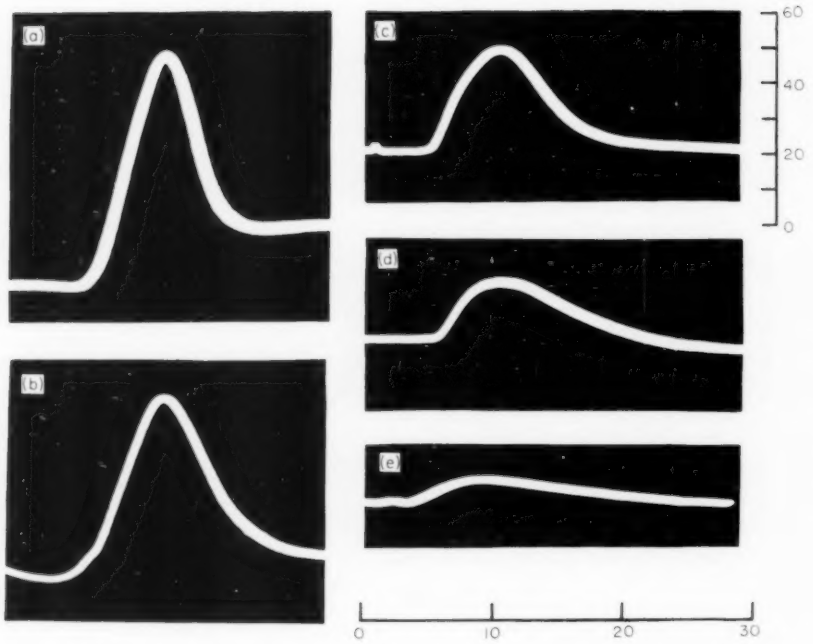

FIG. 4. Effect of sodium ions on locust flexor tibialis. a, in 150; b, 100; c, 50; d, 25; e, zero mM sodium. (a retouched.) Calibration as Fig. 3.

phenomenon occurred, it was exhibited by all the fibres of the muscle under investigation. Occasionally, however, a fibre might show a normal response with a simple *j.p.* after one shock (Fig. 3(a)) and a shock a second later would elicit a response exhibiting a slight hump or plateau (Fig. 3(b)). It seems probable that responses of the type shown in Fig. 3(b), (c) represent a weak but prolonged active membrane response superimposed on the junctional potential, perhaps through leakage of sodium from the fibres.

The time course of the action potential was affected by changes in the external sodium concentration (Fig. 4). As the sodium concentration of the bathing medium was lowered, the rate of rise of the action potential in the cockroach, for example, fell from about 16 V/sec in 150 mM sodium to about 1·4 V/sec in zero sodium saline. There was a corresponding lengthening of the decay phase from a rate of fall of about 16 V/sec in 150 mM sodium to about 0·5 V/sec in zero sodium saline. The rate of rise was found to be directly proportional to the external sodium concentration. The relationship between the rate of decay and the external sodium concentration is more complex, the logarithm of the rate of decay in V/sec being linearly related to the logarithm of the sodium concentration (cases where a hump or plateau was observed in zero sodium salines were ignored when deducing this relationship).

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IV. DISCUSSION

As the sodium concentration in the external medium is progressively lowered, there is a corresponding decrease in the magnitude and rate of rise of the *j.p.* and *a.m.r.* In addition initiation of the *a.m.r.* is delayed. These changes are to some extent in agreement with the effects of sodium ions observed by Fatt & Katz (1952) on the production of the end-plate potential and spike initiation of sartorius muscle fibres in the frog. The two sets of results are not entirely parallel, however. Fatt & Katz (1952) found that in sartorius fibres there is a linear relationship between the size of the end-plate potential and the external sodium concentration, and that its time course was not noticeably altered by changes in sodium concentration. They suggested that sodium ions affected the release of acetylcholine from the motor nerve endings in sartorius fibres. In the locust and cockroach, as in the stick insect (Wood, unpublished observations), the curve relating size of junctional potential to sodium concentration is not linear, but becomes linear when the *j.p.* is plotted against the logarithm of the sodium concentration. Furthermore, the time course of the *j.p.* is considerably lengthened as the external sodium concentration is lowered. The probable explanation for the lengthening of the decay phase is that the withdrawal of sodium ions affects the permeability of the membrane to potassium ions. If there was a general effect of sodium ions which resulted in a decrease in the permeability of the membrane not only to potassium ions but to all ions, including sodium ions, the alteration in the time course of both the rising and decay phases could be due to this single factor. This hypothesis would not exclude an additional effect on the liberation of a transmitter

substance, but the existence of such a substance in insects is at present hypothetical.

The fall in magnitude of the resting potential when sodium ions are withdrawn from the bathing medium is of considerable interest. Interpretation of this result is complicated by the question of how far sucrose and choline may be regarded as 'inert' when substituted for sodium. Fatt & Katz (1953) found that the replacement of sodium chloride by sucrose in the fluid bathing crustacean muscle fibres resulted in a decline in the magnitude of the resting potential from 68 to 38 mV, and the fibres quickly became inexcitable. When choline was substituted for sodium the resting potential again declined, but only by a few mV, and the muscle responses were enhanced. In locust and cockroach muscles, the action of sucrose-substituted and choline-substituted salines is identical. The muscle fibres remain excitable in sucrose-substituted salines, and responses are not enhanced in choline-substituted salines. Although it is not possible to assert that choline and sucrose have no effect on the resting potential, it is equally possible that the action of zero sodium salines reported here is a direct result of the withdrawal of sodium ions from the external medium. If this is so, the effect could again be due to a direct influence of sodium ions on membrane permeability, perhaps because of an effect on membrane structure. It may be noted that Fatt & Katz (1953) suggested that sodium ions may have a 'conditioning' effect on the membranes of crustacean muscle fibres, and Wood (1957) thought that this might also apply to the muscle fibres of *Carausius*.

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COMPARATIVE BIOCHEMICAL STUDIES OF MILKS—I. ELECTROPHORETIC ANALYSIS OF MILK PROTEINS*

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Abstract—1. Paper electrophoretic patterns of caseins and whey proteins from milks of forty species of mammals representing eight orders are presented. Each species has a distinct pattern and related species have similar patterns.

2. Three types of casein patterns were found. The most primitive has a single fast-moving component which is strongly adsorbed by paper. The second type has a prominent slow-moving component and a faster component which is adsorbed. The third type has two sharply differentiated components.

3. In eight species in which whey and blood serum were compared the whey contained components with the mobility of blood serum albumin and gamma globulins.

4. Milks from the primitive orders contained fewer unique whey protein components than milks of more advanced orders.

5. An electrophoretic component tentatively identified as lysozyme appears in whey protein patterns of certain carnivores and perissodactyls.

INTRODUCTION

THIS study represents part of an investigation undertaken to elucidate phylogenetic relationships in mammary gland function as revealed by the composition of milk and the properties of milk constituents. Milks are being collected from as many species as possible, particularly from those of which previous knowledge is meager.

The proteins of all milks previously analyzed consist of a group of phosphoproteins called casein, which is operationally defined as acid precipitable, and the milk serum or whey proteins (Jenness & Patton, 1959; Evans, 1959). In bovine milk the casein is readily separable into three principal fractions designated α, β and γ which differ in composition and properties (Jenness *et al.*, 1956). The α-casein is heterogeneous and has been subfractionated into a number of entities (Brunner *et al.*, 1960). Eight principal components are recognized in the bovine whey proteins (Jenness & Patton, 1959) and numerous "minor proteins" have been described (Whitney, 1958). The proteins in the milks of the goat, sheep, horse, human, pig, rabbit, mouse and rat have been characterized to some extent but by no means as thoroughly as those of the cow (Askonas, 1954; Bain & Deutsch, 1948; Deutsch, 1947; Dovey & Campbell, 1952; Foster *et al.*, 1951; Glass, 1956; Gugler & von Muralt, 1959; Gugler *et al.*, 1958; Hanson, 1959a, b; Hanson & Johansson, 1959; Hofman, 1958a; Johansson, 1954, 1958; Lunsford & Deutsch,

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TABLE 1—CLASSIFICATION OF SPECIES FROM WHICH MILK SAMPLES WERE OBTAINED (AFTER SIMPSON, 1945)

	No. animals*	Total no. milkings	Stage of lactation	Total vol. sample	Remarks	No. other samples*
				(ml.)		
Class Mammalia						
Subclass Theria						
Infraclass Metatheria						
Order Marsupialia						
Family Didelphidae						
Didelphis virginiana	1	1	5 weeks	2·6		
Family Macropodidae						
Setonix brachyurus	1	1	4 months	*ca.* 2		
Macropus rufus	I 1	1	late	50		
	II 1	1	mid	12		
Infraclass Eutheria						
Cohort Unguiculata						
Order Primates						
Family Cercopithecidae						
Macaca mulatta	1	1	mid	16		
Family Hominidae						
Homo sapiens	1	1	4 months	25		3
Order Lagomorpha						
Oryctolagus cuniculus	1	3	mid	28	Pooled	
Order Rodentia						
Family Sciuridae						
Glaucomys volans	1	1	mid	0·6		
Sciurus carolinensis	1	1	late	0·5		
Family Cricetidae						
Subfamily Cricetinae						
Tribe Cricetini						
Mesocricetus auratus	3	5	mid	1·5	Pooled	
Tribe Hesperomyini						
Peromyscus maniculatus	3	3	mid	0·5	Pooled	
Neotoma albigula	1	3	mid	1·5	Pooled	
Subfamily Microtinae						
Tribe Microtini						
Microtus pennsylvanicus	2	3	1 week	0·9	Pooled	
Family Muridae						
Rattus norvegicus	3	3	mid	2	Pooled	
Family Cavidae						
Cavia porcellus	1	4	mid	4	Pooled	1
Family Chinchillidae						
Chinchilla chinchilla	1	3	mid	3·6	Pooled	1
Order Edentata						
Family Dasypodidae						
Dasypus novemcinctus	I 1	1	mid	1·5	Poor lactation	
	II 1	1	late	1·0	Very viscous milk	
Cohort Ferungulata						
Order Carnivora						
Family Procyonidae						
Procyon lotor	5	5	?	4·7	Pooled	
Family Canidae						
Canis familiaris	1	3	mid	23	Pooled	1

TABLE 1—*cont.*

	No. animals*	Total no. milkings	Stage of lactation	Total vol. sample (ml.)	Remarks	No. other samples*
Cohort Ferungulata (*cont.*)						
Family Ursidae						
Ursus horribilis	1	1	late	44		
Family Mustelidae						
Mephitis mephitis	1	1	late	2		
Mustela putorius	1	1	mid	3		1
Family Felidae						
Felis domesticus	1	3	mid	14·5	Pooled	
Panthera leo	1	1	mid	78		
Order Perissodactyla						
Family Equidae						
Equus asinus	1	1	mid	31		
Equus caballus	1	1	early	100	Colostrum	
	1	1	mid	250		4
Equus quagga	1	1	mid	212		
Family Tapiridae						
Tapirus terrestris	1	1	early	120	Colostrum	
Order Artiodactyla						
Suborder Suiformes						
Family Suidae						
Sus scrofa	1	1	1 month			3
Family Tayassuidae						
Tayassu angulatus	1	1	mid	50		2
Suborder Tylopoda						
Family Camelidae						
Lama glama	1	1	mid	165		
Suborder Ruminantia						
Family Cervidae						
Odocoileus virginianus	1	2	mid	40	Pooled	3
Dama dama	1	1	late	25		
Alces canadensis	1	1	mid	110	Freshly killed animal	
Family Antilocapridae						
Antilocapra americana	1	1	late	28	Freshly killed animal	
Family Bovidae						
Subfamily Bovinae						
Bos taurus	—	—	—	—	Pooled herd milk	
Bubalus bubalus	—	—	—	—	Pooled herd milk	
Bison bison	1	1	mid	240		
Subfamily Caprinae						
Capra hircus	—	—	—	—	Pooled herd milk	2
Ovis aries	1	1	mid	140		3
Ovibos moschatus	1	1	mid	105	Freshly killed animal	

* Data given for the samples from which electrophoretic patterns are actually presented. Last column indicates number of additional samples analyzed electrophoretically.

1957; Mellander, 1945, 1947; Moore, 1959; Ross & Moore, 1954, 1955; Schulte & Müller, 1954, 1955). Electrophoretic, ultracentrifugal and immunochemical patterns are characteristic of the species and exhibit marked transitions during the course of the lactation period. Colostrum, secreted immediately following parturition, has a high content of components which appear to be identical to the γ-globulin of blood serum. The content of these proteins decreases to a low level in the first few days of lactation. In the bovine and human species at least, milk contains a small amount of a protein which is identical to blood serum albumin. Apart from the γ-globulins and the serum albumin, the remainder of the principal milk proteins appear to be unique products of the mammary gland.

This paper reports collection of specimens of milk from a number of species and characterization of the milk proteins by the technique of paper electrophoresis.

MATERIALS AND METHODS

Milk samples have been secured to date from forty species representing eight orders of Therian mammals (Table 1). Except as noted they were drawn in the middle portion of the lactation period, and represent normal milk insofar as is known. Some of them were from a single milking of a single female, others were pooled samples. In some cases pentabarbital or ether was used as an anesthetic and oxytocin was often injected to facilitate "let-down". All samples were drawn by manual expression. Milk fat was removed by centrifugation to form a cream layer. Casein was precipitated from the centrifuged milk by acidification to a point of optimum separation, usually pH 4·6, with appropriate dilution in some cases. The precipitated caseins were separated by centrifuging, washed with distilled water and freeze dried. Wheys were dialyzed exhaustively against water to remove the dialyzable fraction which was saved for qualitative analyses of the sugars. The dialyzed whey proteins were freeze dried.

Paper electrophoretic analyses were made on 8 per cent solutions of freeze-dried proteins with the Spinco Model R apparatus. The paper used was S+S 2043 A gl. Caseins were run in veronal buffer at pH 8·6, ionic strength 0·05 for 16 h at 5°C and whey proteins in veronal at pH 8·6, ionic strength 0·075 for 16 h at room temperature. All patterns were stained with brom phenol blue and scanned with the Spinco Analytrol. A casein or whey protein mixture prepared from a composite sample of cow milk was included in each electrophoretic run. This enabled adjustment of the length of patterns to provide comparisons between runs. The integrated curves from the Analytrol were adjusted to a standard concentration.

RESULTS

Figures 1–7 show the paper electrophoretic patterns of the whey and casein proteins. Each scale division represents one centimeter of migration. Commonly used names for the proteins in cow milk are given in Fig. 1. Each peak in a pattern must be interpreted as a potential group of proteins since this has been demonstrated for certain peaks in the case of bovine milk (Brunner *et al.*, 1960).

The whey protein patterns of species of *Bos*, *Capra*, *Ovis*, *Equus*, *Homo*, *Sus*, *Tayassu* and *Rattus* are similar to moving boundary patterns reported in the literature for these species (Deutsch, 1947; Foster *et al.*, 1951; Glass, 1956;

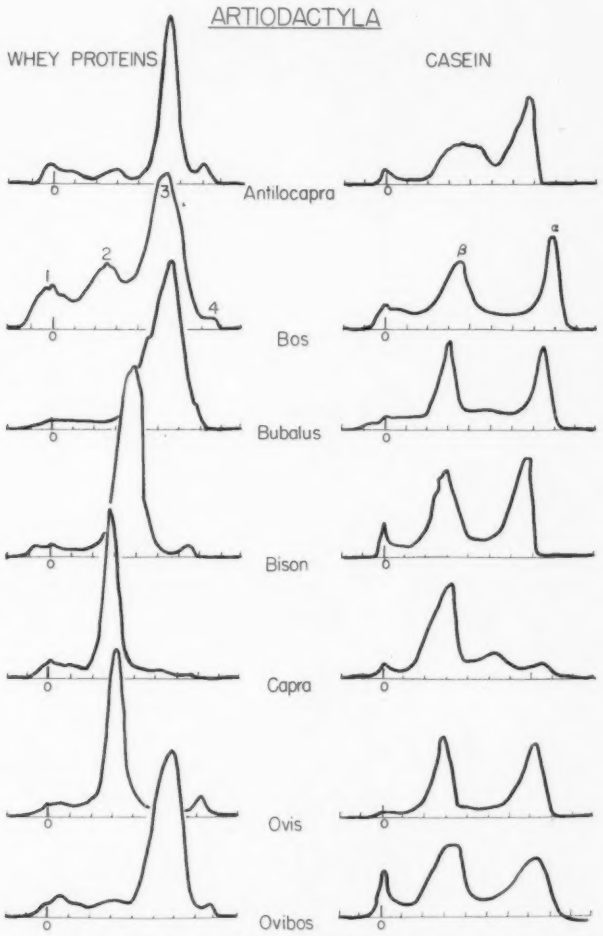

FIG. 1. Electrophoretic patterns of milk proteins of 7 artiodactyls (in whey protein pattern of *Bos*, 1 = immune globulins, 2 = α-lactalbumin, 3 = β-lactoglobulin and 4 = bovine blood serum albumin).

Jenness & Patton, 1959; Lunsford & Deutsch, 1957; and Sowles *et al.*, 1961). Unpublished patterns obtained for the whey proteins of *Bison bison* and *Bubalus bubalus* with the Klett-Tiselius apparatus are very similar to the paper electrophoretic patterns shown in Fig. 1 for these two species. The *Capra*, *Ovis* and

Homo patterns resemble the paper electrophoretic patterns published by Schulte & Müller (1954, 1955).

As indicated in Table 1, specimens from more than one individual animal were analyzed in the case of thirteen species. The individual specimens yielded closely

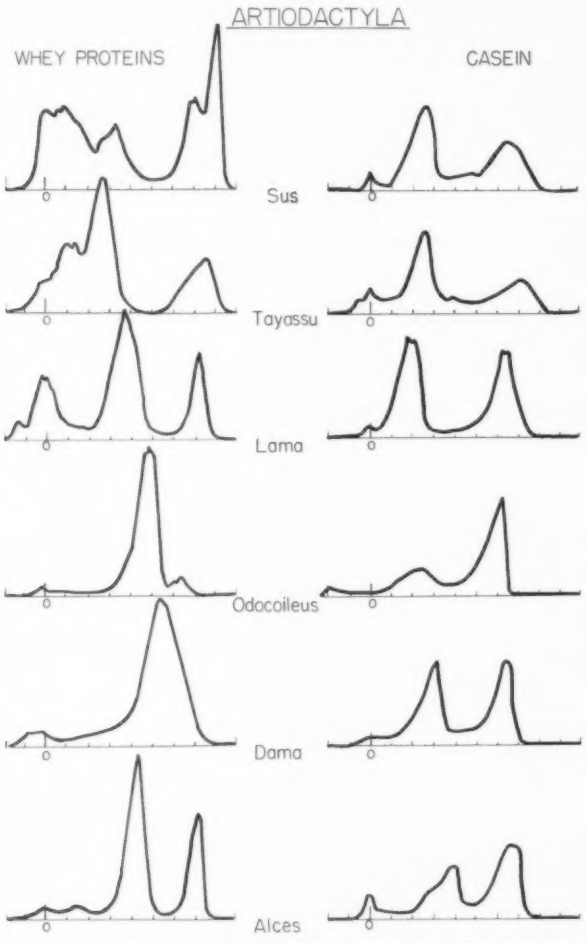

FIG. 2. Electrophoretic patterns of milk proteins of 6 artiodactyls.

similar whey protein patterns except in the case of *Macropus* (both shown in Fig. 7) and *Mustela* (the second specimen, not shown, differed from that in Fig. 4 in that it had a more prominent positively charged component which migrated toward the cathode).

Although no two species have precisely the same pattern, the patterns from several groups of species of tribal to super-family rank exhibit definite similarities.

Fig. 1 shows, somewhat surprisingly, that the whey protein patterns of *Antilocapra* and *Ovibos* closely resemble those of *Bos*. In fact the resemblance is closer than among *Bos*, *Bison* and *Bubalus*. *Capra* and *Ovis*, of course, form a distinctly different group with a prominent component of somewhat lower mobility than the principal component in the other species presented in Fig. 1.

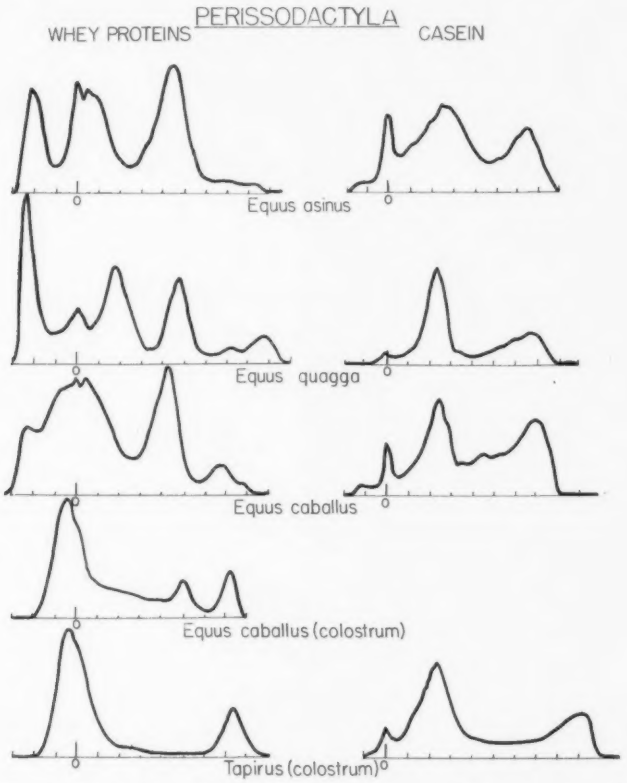

FIG. 3. Electrophoretic patterns of milk proteins of 4 perissodactyls.

The whey proteins of *Odocoileus* and *Dama* are similar (Fig. 2) and resemble those of *Alces* except for the high content of the fast-moving component in the latter which perhaps represents contamination with blood serum albumin from lymph since the specimen was obtained from the excised udder of a road-killed animal. (The specimen was not contaminated with erythrocytes.) The whey proteins of *Sus* and *Tayassu* appear to contain components of similar mobility but in different proportions (see also Sowles *et al.*, 1961).

The three species of *Equus* examined (Fig. 3) appear to have electrophoretically common whey protein components although in somewhat varying proportions.

The colostrum of the tapir (*Tapirus terrestris*), the most primitive surviving perissodactyl, has a very simple whey protein pattern which may be tentatively identified as a mixture of immune globulins and blood serum albumin.

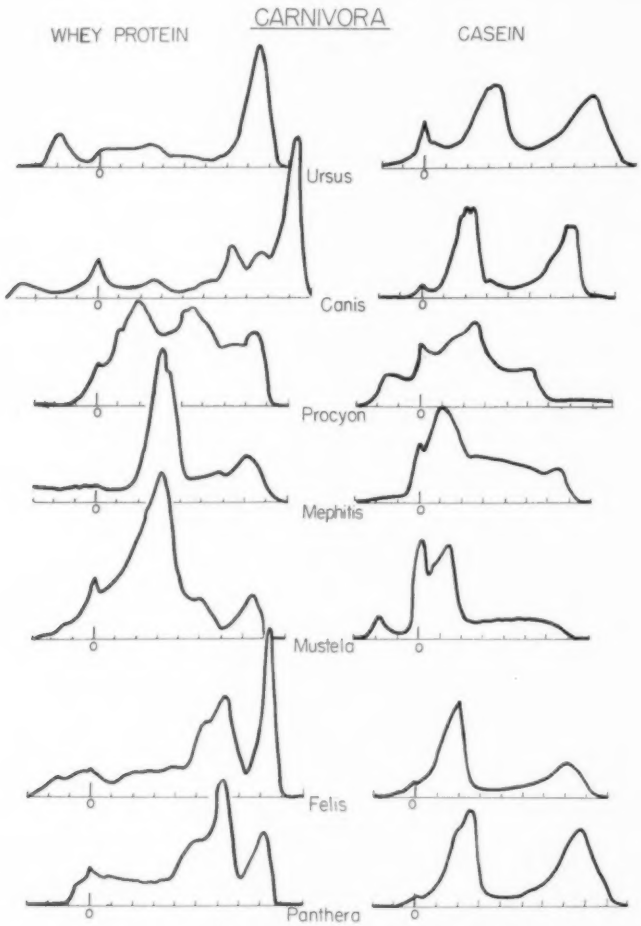

FIG. 4. Electrophoretic patterns of milk proteins of 7 carnivores.

Among the *Carnivora* (Fig. 4) the two mustelids have virtually identical patterns, the two felids are closely similar, *Canis* and *Ursus* are distinct, and *Procyon* has a distinctively individual pattern.

The whey protein patterns from nine rodent species examined (Fig. 5) are distinguishable into four distinct groups representing respectively *Sciuridae*, *Cricetidae*, *Muridae* and *Cavidae*, and *Chinchillidae*.

Figure 6 shows the strong resemblance of the whey protein patterns of *Homo* and *Macaca* (although mobilities differ), the peculiar individuality of the pattern for *Oryctolagus* and the similarity of the two specimens from *Dasypus*.

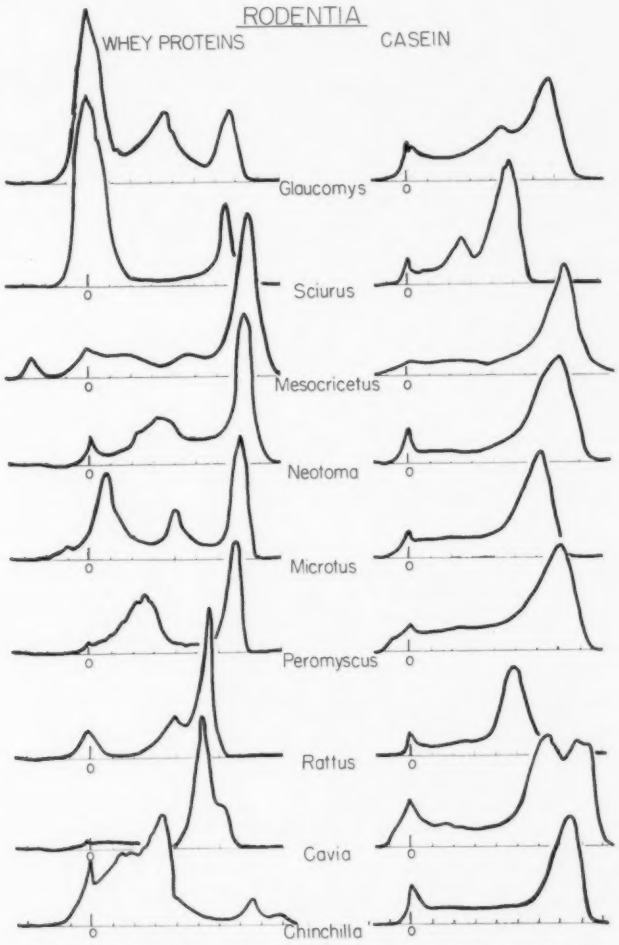

FIG. 5. Electrophoretic patterns of milk proteins of 9 rodents.

The two specimens of *Macropus* milk (Fig. 7) appear to have similar whey protein patterns with some difference in proportions of the components. They differ distinctly from the patterns of *Didelphis* and *Setonix*.

A positively charged component (moving in the opposite direction from the other proteins) is evident in the whey protein patterns of certain species. It is tempting to speculate that this represents the highly basic enzyme lysozyme.

The patterns of *Equus*, *Tapirus*, *Ursus*, *Canis* and *Mustela* all exhibit the positive electrophoretic fraction and have been shown in the literature (Armour & Co., 1952) or by us (unpublished observations) to have lysozyme activity. On the other hand,

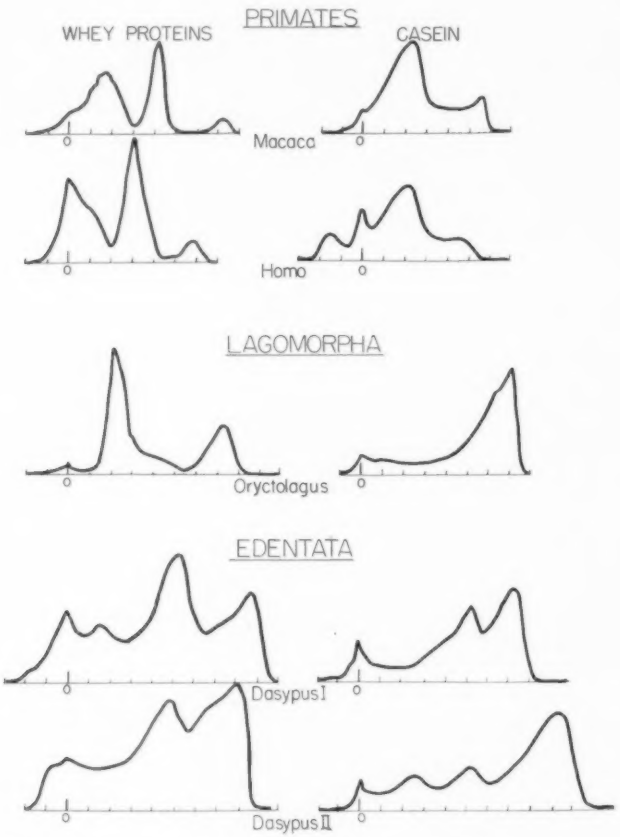

Fig. 6. Electrophoretic patterns of milk proteins of 2 primates, 1 lagomorph and 1 edentate.

the milks of *Homo* and *Sus* are known to possess lysozyme activity, yet they do not exhibit the positively moving component. Cow milk is virtually devoid of lysozyme.

Figures 8 and 9 compare the electrophoretic patterns of the proteins of whey and of blood serum for eight species. Only in the case of *Didelphis* was the blood serum obtained from the same animal which furnished the milk. In every case the whey and blood serum of a species were analyzed on adjacent paper strips. All eight wheys appear to contain components with the mobility of the blood serum albumin and the immune or γ-globulins of blood. The very high concentration

of slow-moving components in whey from *Equus* as compared to blood from this species is distinctive. The blood sera of *Sus* and *Capra* appear to be from hyperimmune animals.

Three principal types of electrophoretic patterns of casein emerge from the study. The first and apparently most primitive, since it occurs in primitive orders, has one peak corresponding in mobility to the high phosphorus or α-casein of cow

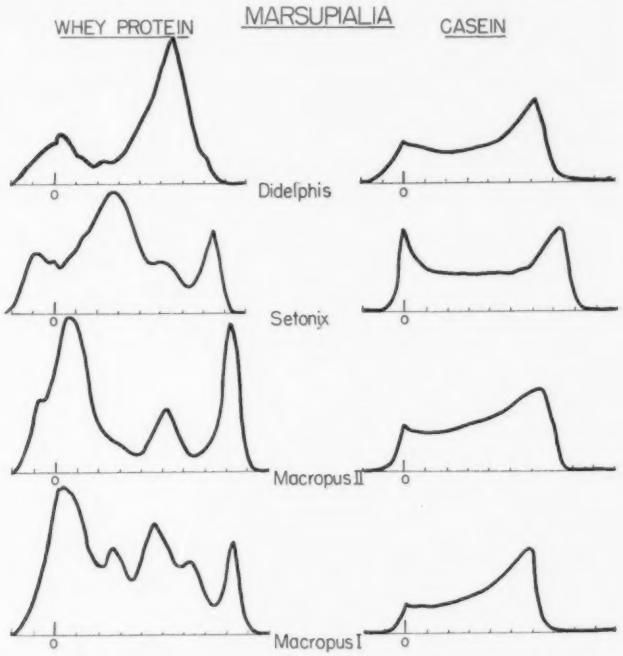

FIG. 7. Electrophoretic patterns of milk proteins of 3 marsupials.

milk. It occurs among the rodents, lagomorphs and marsupials. Moving boundary electrophoresis also yields this type of pattern for the caseins of *Rattus* (Glass, 1956), *Oryctolagus* (Glass, 1956; Dovey & Campbell, 1952), *Mus* (Moore, 1959), *Didelphis* and *Macropus* (unpublished observations).

The second type of casein shows a very prominent slow-moving peak corresponding in mobility to β-casein of cow and a faster-moving component which is greatly adsorbed on the paper. This type of pattern is recognized in *Mustela, Mephitis, Procyon, Macaca* and *Homo.* Of these, only human casein has been analyzed by moving boundary electrophoresis (Glass, 1956; Mellander, 1945); the resulting patterns have two rather distinct components, the faster being in considerably greater concentration than the slower. The peculiar difference between paper and moving boundary patterns for human casein may perhaps be attributed to adsorption of certain components on the paper.

The third general type of casein pattern is characteristic of the balance of the samples and resembles cow casein in having a prominent slow-moving peak corresponding to cow β-casein and a faster-moving peak corresponding to cow α-casein. In *Bos*, there is evidence of a third, more slowly migrating component which probably represents γ-casein; such a component does not appear to any detectable extent in the casein patterns of other artiodactyls. The relative sizes of the two principal casein peaks vary considerably from species to species.

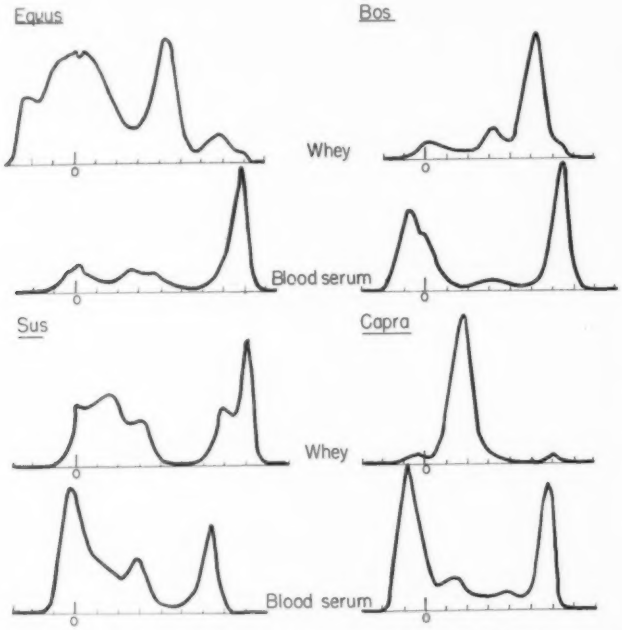

Fig. 8. Comparison of electrophoretic patterns of whey and blood serum of horse, cow, pig and goat.

However, the area of peaks do not necessarily represent accurately the concentration of the "α" and "β" components because of the complicating influences of adsorption and differences in dye-binding capacity. Moving boundary patterns (at 1·5 per cent protein) for the caseins of *Bos*, *Bison* and *Bubalus* (unpublished) revealed a higher ratio of α/β than exhibited by the paper patterns. In moving boundary patterns of *Sus* and *Tayassu* caseins (Sowles *et al.*, 1961) the faster component was split into two peaks.

Casein patterns of specimens from individual animals of a species were always very similar except in the case of *Dasypus* for which both patterns obtained are depicted in Fig. 6. The caseins of two species of the *Sciuridae* conform to the general rodent pattern of a single fast-moving peak which, however, shows some tendency to split into two peaks. Likewise goat casein is a special case of the third

type of casein pattern since the faster peak is split into two distinguishable components as has also been demonstrated with paper patterns by Hofman (1958a).

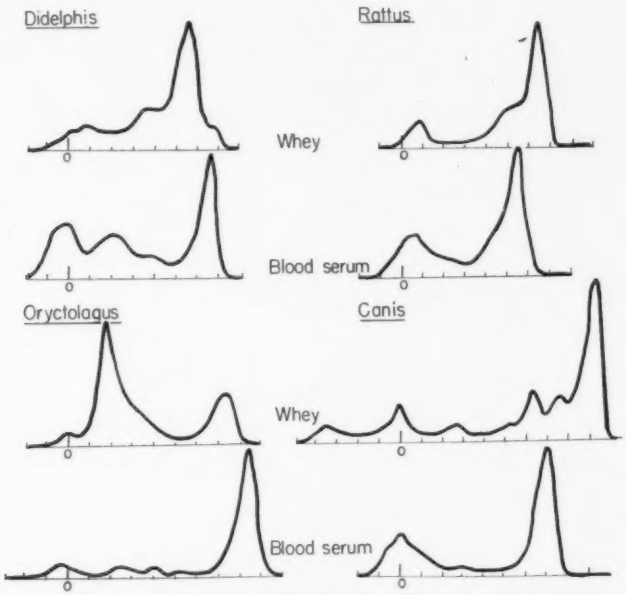

FIG. 9. Comparison of electrophoretic patterns of whey and blood serum of opossum, rat, rabbit and dog.

DISCUSSION

In general, primitive surviving stocks of any order of mammals have fewer quantitatively important milk proteins than phylogenetically more recent derivatives. The Rodentia and Lagomorpha have the fewest electrophoretically distinguishable milk proteins of the orders studied with four the most frequent number. A comparison of the electrophoretic patterns of the whey proteins with those of blood serum indicated that in the relatively simple milks of the Rodentia and Lagomorpha the whey proteins also appear to occur in the blood serum (although in some cases certain blood serum proteins apparently do not occur in the whey). The patterns of the caseins show that only one principal distinguishable component is present in the milks of rabbits and most rodents. This single component corresponds in mobility to the α-casein (high phosphorus component) of cow casein. It is difficult to conceive of a milk of much more simple protein composition than in these primitive and generalized orders. The marsupials investigated, like the Rodentia and Lagomorpha, have only one casein, corresponding to cow α-casein, but have additional whey proteins not present in the blood serum as in phylogenetically more advanced placentals. The advanced

Primates and two rodents have two principal caseins which presumably represent independent acquisitions.

The electrophoretic mobilities of casein fractions are undoubtedly partly determined by the content of ester-bound ionizable phosphate groups. Thus bovine α-casein has 24 more phosphate groups and 24 more carboxyl groups per 100,000 g than does bovine β-casein (Jenness & Patton, 1959). Table 2 summarizes available data from the literature on the P/N ratio of caseins of various species. The caseins of *Rattus* and *Mus*, consisting of a single component of high

Table 2—Phosphorus–nitrogen ratios of caseins of various species

Species	P/N Wt. ratio	Reference
Bos taurus α-casein	0·064	Jenness & Patton, 1959
β-casein	0·043	Jenness & Patton, 1959
Whole	0·055	Jenness & Patton, 1959
Capra hircus α-casein	0·065	Hofman, 1958b
β-casein	0·040	Hofman, 1958b
Whole	0·049	Davies, 1939
Ovis aries Whole	0·052	Davies, 1939
Equus caballus Whole	0·054	Davies, 1939
Equus asinus Whole	0·065	Davies, 1939
Rattus norvegicus Whole	0·062	Glass, 1956
Mus musculus Whole	0·078	Ross & Moore, 1955
Oryctolagus cuniculus Whole	0·052	Davies, 1939
Homo sapiens α-casein	0·063	Mellander, 1945
Whole	0·030	Mellander, 1945
Whole	0·046	Davies, 1939

mobility, have a high content of phosphorus. That of *Oryctolagus*, which also has one component of high mobility, is not especially high in phosphorus. In species having more than one principal component the one of greater mobility has a higher phosphorus content. Of course any generalizations regarding phosphorus content and its relation to mobility must await more extensive analysis for this element in the caseins.

All milks appear to contain the phosphoproteins commonly termed "caseins". These are of obvious nutritional value to the mammalian young, not only because they carry phosphorus, but also because they form colloidal complexes with calcium. The origin of the caseins is thus a problem of immense significance. Marston (1926) demonstrated the presence in the milk of the monotreme, *Echidna aculeata multi-aculeata*, killed 14 days after removal of the young, of a protein

precipitable at pH 4·7 which is presumably a "casein". It represented 8·4 per cent of the milk and 74 per cent of the total protein. If the monotremes do indeed secrete such characteristic milk constituents as casein an interesting problem is posed since the most recent common ancestry of the monotremes and Therian mammals is apparently early in the history of therapsid reptiles (Brink, 1957; Olson, 1959; Patterson, 1956; Romer, 1956; Watson & Romer, 1956). Thus the ability to synthesize casein must have been acquired independently in the Therian and monotreme ancestry or casein (and thus milk) was secreted by the therapsids themselves. It would, of course, be of tremendous interest to analyze a specimen of monotreme milk by modern methods.

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CARBOHYDRATE ABSORPTION IN CESTODES FROM ELASMOBRANCH FISHES*

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Abstract—Eight species of cestodes from elasmobranch fishes absorbed glucose and galactose. *Lacistorhynchus tenuis* and *Phyllobothrium foliatum* absorbed maltose readily, and the latter took up this disaccharide much more rapidly than glucose. Several glycosides and other phenolic derivatives exerted a predominantly inhibitory effect on the carbohydrate consumption by the worms. However, naringenin, phlorizin, and rutin accelerated the rate of glucose uptake by *Onchobothrium pseudo-uncinatum*, and a similar result was obtained by exposing *Calliobothrium verticillatum* to a medium containing *p*-(2,4-dihydroxyphenylazo)benzene sulfonic acid. Quercetin, diethylstilbesterol, *p,p'*-biphenol, and bis(*p*-hydroxyphenyl) methane did not block glucose consumption by *C. verticillatum* and *L. tenuis*.

The worms contained at least three carbohydrate fractions, two of which were stable to alkaline digestion. One of the alkali-stable fractions precipitated in 50 per cent aqueous ethanol, whereas the other did not do so. *Inermiphyllidium pulvinatum*, *L. tenuis*, and *O. pseudo-unicinatum* synthesized a net increase in polysaccharide during four hours' incubation in the presence of glucose.

A substance that was presumably lactic acid was excreted by all eight species of cestodes.

INTRODUCTION

THE present study constitutes an extension of previously reported work concerning the penetration of carbohydrates into helminths obtained from mammalian hosts (Laurie, 1957, 1959). In some of their morphological characteristics the members of the Tetraphyllidea and the Trypanorhyncha differ conspicuously from mammalian cestodes. Whether this applies to their physiological characteristics remains, for the most part, unknown. Read, Douglas, & Simmons (1959) have demonstrated that the presence of urea is detrimental to several species of platyhelminths that do not normally inhabit environments containing this substance. The permeation of urea and amino acids into cestodes taken from elasmobranches, as well as into host tissues, has been studied by Read and his associates (Simmons, Read, & Rothman, 1960; Read, Simmons, Campbell, & Rothman, 1960; Read, Simmons & Rothman, 1960).

The data reported herein were obtained, at such times as infected hosts became available, during the summers of 1958, 1959, and 1960.

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MATERIALS AND METHODS

From a consideration of the feeding habits of the hosts, it was decided that the most likely carbohydrates being presented to the inhabitants of the spiral valve would be glycogen and its degradation products, especially maltose and glucose. This point, and results of earlier work with dogfish tapeworms (Read, 1957) and helminths from rats and reptiles (Laurie, 1957, and unpublished data), served as a basis for restricting the number of substrates employed. Phlorizin was selected as a reference inhibitor, and a number of other glycosides and phenolic derivatives were tested for possible inhibitory actions.

Lacistorhynchus tenuis and *Calliobothrium verticillatum* were obtained from the Smooth Dogfish (*Mustelus canis*); *Onchobothrium pseudo-uncinatum*, *Phyllobothrium foliatum*, *Anthobothrium variable*, and *Inermiphyllidium pulvinatum* from the Northern Sting-ray (*Dasyatis centrura*); *Orygmatobothrium dohrnii* (Oerley, 1885) Riser, 1955 from the Sand Shark (*Carcharias taurus*); and *Disculiceps pileatum* from the Dusky Shark (*Carcharhinus obscurus*). The generic designations only will be used below. Host identifications were based on the monograph by Bigelow and Schroeder (1954); while various papers by Linton (see 1924) and Riser (1955) were used extensively for making parasite identifications. *Lacistorhynchus* represented the Order Trypanorhyncha, and the other species are usually considered to be members of the Order Tetraphyllidea.

The spiral valve was removed from the host as soon as the latter arrived at the dock, and was subsequently slit open and examined for the presence of the appropriate tapeworms. Those present were removed, freed of debris, and placed in a suitable elasmobranch Ringer's solution. These solutions contained the following materials: sodium chloride, potassium chloride, calcium chloride, and magnesium chloride present in the amounts of 190, 4·4, 5·1, and 2·9 mmoles/l., respectively. In addition, the shark Ringer's contained 0·3 M urea and the ray Ringer's 0·4 M urea (Simmons, personal communication). The Ringer's solutions were buffered at a pH of 7·2 with a 0·01 M tris(hydroxymethyl)aminomethane-maleic acid mixture (Colowick & Kaplan, 1955).

Substrate concentrations were equivalent to 0·002 M or 0·005 M hexose. Unless otherwise indicated, by figures enclosed in parentheses, the various phenolic compounds were used at a concentration equivalent to $5{\cdot}73 \times 10^{-5}$ M phlorizin. The sulfone polymer was made from 4,4′-dihydroxydiphenylsulfone and phosphoryl chloride by employing the techniques of Diczfalusey and his associates (1953). After stopping the polymerizing reaction, the mixture was transferred to a cellophane bag and dialyzed against several changes of distilled water. The fraction remaining in the bag was recovered, dried, and tested for its possible inhibitory properties.

In the absence of an inhibitor, the worms were incubated aerobically at 20°C for 4 h in 3·0 ml of medium containing 0·005 M hexose, or its equivalent. In the media containing phenolic substances the carbohydrate substrate was decreased to a concentration equivalent to 0·002 M hexose, the volume of medium was decreased to 2 ml, and the incubation time was shortened to one hour. When not otherwise

indicated by subscripts, *Lacistorhynchus* and *Calliobothrium* were incubated in groups of four to eight individuals, *Phyllobothrium* in groups of 2–3 individuals, and *Anthobothrium* in groups of 2–4 individuals. These groups attained live weights of approximately 10–50 mg. The other cestodes routinely weighed over 50 mg, and thus were incubated singly. The numbers of groups involved in the inhibition studies are usually designated by figures enclosed in parentheses and lacking subscripts.

Following incubation, the worms were stored in 95 per cent aqueous ethanol if they were to be analyzed for their carbohydrate content or placed in an oven and dried to a constant weight at 90°C. The media were frozen and later analyzed for their carbohydrate and (presumably) lactic acid content. Mokrasch's anthrone reagent (1954) was employed to measure carbohydrate; but his procedure was modified to the extent that glucose, maltose, and mannose were heated for 44 min, galactose for 40 min, and fructose for 4 min. The procedure can be greatly expedited if the anthrone reagent is chilled to 0° or lower, and then syringed rapidly into the solution containing the carbohydrate sample. The amount of a material assumed to be lactic acid was estimated by the procedure of Barker & Summerson (1941). For tissue analyses the ethanol was evaporated at 45°C, and the tissue was then homogenized in cold (5°C), 0·01 N sulfuric acid. An aliquot was heated at 80°C for 30 min with an equal volume of 30 per cent potassium hydroxide. Subsequent treatments of the tissue sample followed the procedure described elsewhere (Laurie, 1959). For ascertaining the dry weight of the tissue one third to one half of the homogenate was dried to a constant weight at 90°C.

In some preliminary experiments involving host tissue, small pieces of spiral valve, with mucosa on both surfaces, were removed from the middle portion of this organ.

The results are presented in Tables 1 and 2. If several groups of worms were available the means are accompanied by their respective standard deviations. Differences between mean values were compared with the aid of Student's "t" test and were considered to be significant when $P < 0{\cdot}05$.

RESULTS

Except for *Phyllobothrium*, all the worms removed glucose from the environment at a rate that noticeably exceeded the rate of maltose uptake (Table 1). *Phyllobothrium* exhibited a striking difference in the relative rates of absorption of these two substrates in that maltose entered approximately ten times more rapidly than glucose. The response of *Calliobothrium* to the presence of maltose was peculiar since a carbohydrate, or carbohydrate-like substance, leaked out of the organism and/or was displaced from its surface. Hence, the amount of material in the medium that reacted with the anthrone reagent increased during the incubation period. Four species took up galactose about as readily as glucose, but in the remaining species, the latter substrate was consumed more rapidly. Small amounts of fructose and mannose penetrated into *Calliobothrium* and *Disculiceps*,

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TABLE 1—CARBOHYDRATE UPTAKE AND LACTIC ACID EXCRETION IN μg/mg DRY WT./HR BY ELASMOBRANCH HELMINTHS

Substrates	*Calliobothrium*	*Lacistorhynchus*	*Onchobothrium*	*Phyllobothrium*	*Antho-bothrium*	*Inermi-phyllidium*	*Orygmato-bothrium*	*Disculiceps*	*Mustelus* spiral valve	*Dasyatis* spiral valve
Glucose	$20{\cdot}4 \pm 2{\cdot}0(5_3)$[a]	$32{\cdot}9 \pm 1{\cdot}2(4_7)$	$7{\cdot}1 \pm 3{\cdot}1(5_1)$	$1{\cdot}6 \pm 1{\cdot}4(4_3)$	$20{\cdot}1(2_4)$	$28{\cdot}3(2_1)$	$6{\cdot}9 \pm 2{\cdot}2(4_3)$	$3{\cdot}1(2_1)$	$2{\cdot}3 \pm 1{\cdot}2(11)$	$1{\cdot}1(2)$
Lactic acid	$0{\cdot}42 \pm 0{\cdot}17$†	$0{\cdot}80 \pm 0{\cdot}25$*	$1{\cdot}02 \pm 0{\cdot}66$*	$1{\cdot}44 \pm 0{\cdot}30$†	$0{\cdot}8$	$1{\cdot}1$	$0{\cdot}30 \pm 0{\cdot}30$	$0{\cdot}05$	$1{\cdot}63 \pm 0{\cdot}12$	—
Glucose cont.[b]	$21{\cdot}1 \pm 5{\cdot}4(15)$	$32{\cdot}7 \pm 7{\cdot}8(22)$	$6{\cdot}2 \pm 2{\cdot}4(11)$	—	—	—	—	—	—	—
Galactose	$19{\cdot}6 \pm 4{\cdot}5(9_3)$	$17{\cdot}0 \pm 1{\cdot}8(7_3)$	$8{\cdot}8 \pm 1{\cdot}1(6_1)$	$2{\cdot}0 \pm 1{\cdot}3(4_3)$	$8{\cdot}5(3_3)$	$4{\cdot}5 \pm 1{\cdot}3(3_1)$	$7{\cdot}1 \pm 3{\cdot}2(4_3)$	—	$0{\cdot}8 \pm 0{\cdot}2(4)$	—
Lactic acid	$0{\cdot}30 \pm 0{\cdot}07$†	$0{\cdot}21 \pm 0{\cdot}09$	$0{\cdot}42 \pm 0{\cdot}11$	$1{\cdot}24 \pm 0{\cdot}11$†	$0{\cdot}5$	$0{\cdot}11 \pm 0{\cdot}04$	$0{\cdot}12 \pm 0{\cdot}05$	—	$1{\cdot}18 \pm 0{\cdot}22$	—
Fructose	$0{\cdot}5(2_3)$	0 (Read, 1957)	$0(4_1)$	$0(2_3)$	—	$0(4_1)$	—	$0(2_1)$	—	—
Lactic acid	$0{\cdot}3$	—	$0{\cdot}4$	$0{\cdot}7$	—	$0{\cdot}03$	—	$0{\cdot}1$	—	—
Mannose	0 (Read, 1957)	0 (Read, 1957)	$0(4_1)$	$0(8_3)$	—	—	—	$0{\cdot}2(4_1)$	—	—
Lactic acid	—	—	$1{\cdot}0$	$0{\cdot}70 \pm 0{\cdot}10$	—	—	—	$0{\cdot}04$	—	—
Maltose	$-2{\cdot}6$[c](3_3)	$10{\cdot}6(3_3)$	$0(4_1)$	$16{\cdot}7 \pm 1{\cdot}4(7_3)$	—	$0{\cdot}6(2_1)$	—	$0(3_1)$	—	—
Lactic acid	$0{\cdot}1$	$1{\cdot}29 \pm 0{\cdot}18$†	$0{\cdot}5$	$0{\cdot}46 \pm 0{\cdot}14$	—	$0{\cdot}05$	—	$0(3)$	—	$0{\cdot}9(2)$
Buffer	—	—	—	—	—	—	—	—	—	$0{\cdot}9$
Lactic acid	$0{\cdot}12 \pm 0{\cdot}13(6_3)$	$0{\cdot}52 \pm 0{\cdot}08(6_3)$	$0{\cdot}30 \pm 0{\cdot}20(7_1)$	$0{\cdot}87 \pm 0{\cdot}17(12_3)$	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	$1{\cdot}5(2)$	$0{\cdot}6(2)$
Phlorizin (150)	0†(5)[d]	$-5{\cdot}6$†(4)	$1{\cdot}1$†(5)	—	$2{\cdot}4$†(3)	$0(1_1)$	—	—	—	$0{\cdot}6(2)$
Glucose	—	—	—	—	—	—	—	—	—	—
Phlorizin (100)	$-0{\cdot}3$†(6)	$0{\cdot}9$†(6)	$0{\cdot}3$†(6)	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Phlorizin (25)	$-2{\cdot}4$†(9)	—	$11{\cdot}6$†(5)	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Phlorizin (15)	$-1{\cdot}8$†(3)	—	—	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Phlorizin (1)	$13{\cdot}1$*(3)	$25{\cdot}9(3)$	—	—	—	—	—	—	$0{\cdot}4 \pm 0{\cdot}2$*(4)	$1{\cdot}1(2)$
Galactose	—	—	—	—	—	—	—	—	—	—
Phlorizin (150)	0(4)	$-7{\cdot}5(4)$	$<0{\cdot}1(5)$	—	—	$0{\cdot}3(1_1)$	—	—	0(4)	—
Glucose	—	—	—	—	—	—	—	—	—	—
Phloretin	$11{\cdot}3$†(3)	$23{\cdot}0$*(5)	$14{\cdot}0$†(6)	—	—	—	—	—	—	—
Glucose	$27{\cdot}7(3)$	—	—	—	—	—	—	—	—	—
Quercitrin	$(P<0{\cdot}1)$	$29{\cdot}2(3)$	—	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Rutin	$3{\cdot}1$†(3)	$21{\cdot}2$*(3)	$14{\cdot}8$†(4)	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Eastman 1992[e]	$30{\cdot}8$†(3)	$31{\cdot}6(3)$	—	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Naringenin	$10{\cdot}4$*(3)	—	$17{\cdot}0$†(5)	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Polymer (150)	$15{\cdot}2$*(9)	$11{\cdot}2$†(4)	$2{\cdot}8$*(5)	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Polymer (100)	$11{\cdot}6$*(3)	—	—	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Polymer (50)	$14{\cdot}8$*(3)	—	—	—	—	—	—	—	—	—
Galactose	—	—	—	—	—	—	—	—	—	—
Polymer (150)	$3{\cdot}9$†(5)	$28{\cdot}4$*(4)	$5{\cdot}7$†(4)	—	—	—	—	—	—	—
Glucose	—	—	—	—	—	—	—	—	—	—
Indophenol	$9{\cdot}6$†(3)	—	—	—	—	—	—	—	—	—

a. Five groups of 8 individuals per group, etc.
b. Control rates which were compared with the rates when phlorizin, etc., were present.
c. A minus sign indicates that the material reacting with the anthrone reagent increased during the incubation.
d. See the text for an explanation of the figures in parentheses lacking subscripts.
e. *p*-(2,4-dihydroxyphenylazo)benzene sulfonic acid.
* $P<0{\cdot}05$. † $P<0{\cdot}01$.

respectively; whereas the other worms did not take up either of these hexoses (Table 1).

The worms excreted a substance that presumably was lactic acid, and, in general, its excretion rate in media containing carbohydrates that were taken up exceeded the excretion rate in the absence of an exogenous substrate. Typically, the stomachs of the hosts contained fish in various stages of digestion. Since it was

TABLE 2—CARBOHYDRATE CONTENT OF ELASMOBRANCH HELMINTHS AS PER CENT OF THE DRY WEIGHT

Substrates	*Callio-bothrium*	*Lacisto-rhynchus*	*Oncho-bothrium*	*Phyllo-bothrium*	*Inermi-phyllidium*	*Disculiceps*
Glucose	— $5{\cdot}4 \pm 1{\cdot}6(5_8)$ $1{\cdot}9 \pm 0{\cdot}9$	$8{\cdot}1 \pm 0{\cdot}6(4_7)$ $1{\cdot}1 \pm 0{\cdot}4$* $2{\cdot}3 \pm 0{\cdot}3$	$22{\cdot}9 \pm 1{\cdot}6(5_2)$ $18{\cdot}3 \pm 2{\cdot}9$* 1·7	$9{\cdot}1(2_7)$ 3·1 1·7	$26{\cdot}9 \pm 2{\cdot}8(3_1)$ $23{\cdot}4 \pm 4{\cdot}8$* 1·2	$11{\cdot}8(2_1)$ 9·1 0·8
Galactose	6·1[a](2_{10}) 0[b] 1·3[c]					
Fructose				$10{\cdot}3(2_3)$ 8·0 —	$13{\cdot}4 \pm 1{\cdot}1(4_1)$ $10{\cdot}8 \pm 3{\cdot}2$ $1{\cdot}1 \pm 0{\cdot}5$	$14{\cdot}1(2_1)$ 11·1 1·7
Maltose						$12{\cdot}6 \pm 1{\cdot}3(3_1)$ $10{\cdot}8 \pm 1{\cdot}5$ $1{\cdot}3 \pm 0{\cdot}4$
Buffer	$7{\cdot}6 \pm 1{\cdot}5(6_5)$ $4{\cdot}2 \pm 2{\cdot}0$ $2{\cdot}2 \pm 0{\cdot}3$	$3{\cdot}5 \pm 0{\cdot}6(6_6)$ $0{\cdot}02 \pm 0{\cdot}4$ $2{\cdot}6 \pm 0{\cdot}3$	$13{\cdot}7 \pm 1{\cdot}1(7_1)$ $8{\cdot}0 \pm 1{\cdot}8$ $4{\cdot}8 \pm 2{\cdot}2$	$15{\cdot}0 \pm 2{\cdot}5(12_3)$ $9{\cdot}5 \pm 2{\cdot}4$ $4{\cdot}1 \pm 2{\cdot}4$		

a. Total carbohydrate.
b. Polysaccharide.
c. Alkali-stable, ethanol-soluble carbohydrate.
* $P<0{\cdot}01$.

impractical to retain the hosts in a fasting condition for several days, some of the endogenous metabolic activities of the worms may be much greater than would be the case if the worms had been recovered from starved hosts.

The absorption of glucose and fructose could not be demonstrated for *Entobdella bumpusi*, a monogenetic trematode found attached to the skin of *Dasyatis*. Glucose slowly entered (0·2 μg/mg live wt./h) the aspidobothrian *Macraspis cristata* (see Dolfus, 1958), an inhabitant of the gall bladder of *Dasyatis*; whereas maltose, and apparently galactose, did not do so.

The presence of the glycosides and other phenolic derivatives in the environment evoked varied responses from the different species of worms. Glucose absorption in *Calliobothrium* and *Lacistorhynchus* was unaffected by *p,p'*-biphenol, bis(*p*-hydroxyphenyl)methane, diethylstilbesterol, and quercetin.

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Phlorizin ($3{\cdot}44 \times 10^{-4}$ M), quercitrin, and rutin reduced the rates of maltose uptake by *Phyllobothrium* to 0·6, 4·8, and 10·1 μg/mg dry wt./h, respectively, a significant inhibition ($P < 0{\cdot}01$) in each case. As a rule the phenolic substances tended to inhibit the rate of carbohydrate uptake (Table 1). However, in the case of *Onchobothrium*, naringenin, phloretin, phlorizin, and rutin accelerated the rate of glucose uptake by this worm. This may apply to *Calliobothrium* when it is in contact with quercitrin, and does apply when it is exposed to *p*-(2,4-dihydroxyphenylazo)benzene sulfonic acid (Eastman Cat. No. 1992). The "leakage" phenomenon mentioned above also occurred when *Calliobothrium* and *Lacistorhynchus* were exposed to several concentrations of phlorizin. Preliminary experiments with *Mustelus* and *Dasyatis* spiral valve tissue indicate that these tissues differ from each other in their sensitivity to the presence of phlorizin.

The carbohydrate content of the worms can be divided into at least three fractions, one of which is unstable to alkaline hydrolysis, the other two being stable to this treatment. One of the stable fractions precipitates in 50 per cent aqueous ethanol and is very likely a glycogen. The other stable fraction is soluble in this alcoholic solution. This could be a mixture of oligosaccharides or possibly trehalose, a disaccharide that has been shown to be present in numerous invertebrates, including several helminths (Fairbairn, 1958). The amount of the alkali-stable, alcohol-soluble fraction tended to be similar in the different species of worms examined. However, this did not apply to the polysaccharide fraction, which was distinctly higher in *Onchobothrium* and *Inermiphyllidium*, as compared to the other species, and conspicuously lower in *Lacistorhynchus*. Specimens of *Lacistorhynchus*, *Onchobothrium*, and *Inermiphyllidium* incubated in the presence of glucose for four hours contained appreciably greater amounts of polysaccharide than those that were incubated in the buffer only or in the presence of fructose. This difference apparently represents a net synthesis of polysaccharide during a 4-hr period, although some of it may have been synthesized before the worms were removed from the hosts.

DISCUSSION

The removal of glucose and galactose from the environment at rates that usually exceed the uptake of other carbohydrates is not peculiar to the elasmobranch cestodes, since this characteristic has been exhibited by helminths from mammalian hosts (see Read, 1959). The use of a disaccharide at a rate that distinctly surpasses the rates at which the monosaccharide components are utilized has been observed in *Cittotaenia* sp., a cestode from rabbits (Read & Rothman, 1958); thus this phenomenon is not unique to *Phyllobothrium*. *Phyllobothrium* consistently inhabits the last quarter, or less, of the host's spiral valve. If one assumes that most, if not all, of the free glucose formed by the digestive activities of the host is absorbed in the anterior regions of the spiral valve, then any helminth inhabiting the posterior end of this organ would be deprived of this source of carbohydrate. The available data suggest that *Phyllobothrium* possesses a specific transporting mechanism for removing maltose from the external medium. The ability to take

up this disaccharide rapidly may be the critical factor that enables this worm to persist in the region where it is invariably located. The other cestode species found in *Dasyatis*, including some not used for experimental purposes, were distributed in what seemed to be a non-random manner in the anterior three quarters of the spiral valve, e.g., *Onchobothrium* tended to be localized in the middle two quarters. No explanation can be offered to account for the response of *Calliobothrium* to the presence of maltose. Campbell (1960) has reported on the presence of β-amino acids in *Macraspis*, but the rarity of this organism precluded its use to any extent for experimental purposes.

The polysaccharide content of *Calliobothrium*, *Lacistorhynchus*, and *Phyllobothrium* was much lower than the amounts reported for cestodes recovered from mammals (von Brand, 1952). This must be an innate characteristic of these worms and not the result of inhabiting an environment of relatively low temperature, since *Onchobothrium* and *Inermiphyllidium* both contained large amounts of polysaccharide. The failure to demonstrate a net synthesis of the alkali-stable, ethanol-soluble carbohydrate fraction could be anticipated from similar results obtained earlier while working with a mammalian helminth (Laurie, 1959).

Prior work demonstrated that one could expect some phenolic compounds to interfere in carbohydrate absorption by cestodes (Laurie, 1957). The results obtained to date with the elasmobranch tapeworms support the following conjectures: the presence of the carbohydrate moiety enhances the inhibitory activity of phlorizin, quercitrin, and rutin, and its absence may lead to a complete loss of activity as it did with quercetin, the aglycon of the last two glycosides. In the absence of a flavone configuration, a minimum spacing of about 13 Å between the para-situated, hydroxyl groups of phlorizin and its aglycon, phloretin, may account for part of the inhibitory property of these two substances. The phenolic derivatives with hydroxyl groups similarly located, but less widely spaced, were inactive. It is not clear whether the inhibitions involve a transporting mechanism, the subsequent metabolism of the substrate in question, or a combination of these factors, but experiments concerned with the inhibition of glucose uptake in *Hymenolepis diminuta* suggest that the inhibition may involve the former process and not the latter (Laurie, 1957). No explanation can be offered to account for the stimulation of glucose uptake by *Calliobothrium* and *Onchobothrium* when they were in contact with some of the phenolic compounds. The dimensions of the sulfone polymer may be such that this substance exerts its inhibitory effect by adsorbing to the surface of the worm; however, since its exact size is not known, the possibility that it might penetrate into the worm and function intracellularly cannot be excluded from consideration.

The predominantly adverse effects of the various phenolic derivatives on the metabolic activities of the elasmobranch cestodes, and the much greater sensitivity of these organisms to phlorizin as compared to the mammalian helminths worked with previously (Laurie, 1957), may represent one of the mechanisms whereby the distribution and survival of helminths in different hosts are controlled. If the host ingests food material, continuously or seasonally, containing a substance that

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interferes with the metabolic activities of the parasite, the latter may be unable to infect, or survive in, the host during this time. Alternatively, the response of the parasite may be quantitative in that smaller individuals, and fewer of them, will survive in the host when the latter's diet contains materials that are inimical to the parasite. If this supposition is correct, one might expect helminths from carnivorous hosts to be more susceptible to metabolic interference by phenolic glycosides, or other phenolic compounds, than those species that live in herbivorous or omnivorous hosts. The results obtained with the helminths recovered from mammals (Laurie, 1957) and elasmobranch fishes partially support this contention. Also, phlorizin ($4{\cdot}58 \times 10^{-4}$ M) reduced the rate of glucose uptake by *Ophiotaenia marenzelleri*, a cestode parasite of the Cottonmouth (*Ancistrodon piscivorus*), to less than 5 per cent of the control rate (unpublished data). I have examined, or observed, specimens of this snake containing, or feeding on, fish, frogs, and rodents and it appears that this host feeds exclusively on various small vertebrates (Wright & Wright, 1957).

SUMMARY

Eight species of tapeworms from elasmobranch fishes absorbed glucose and galactose. *Lacistorhynchus tenuis* and *Phyllobothrium foliatum* also absorbed maltose readily, and the latter took up this disaccharide more rapidly than glucose.

Several glycosides and other phenolic derivatives exerted a predominantly inhibitory effect on carbohydrate uptake by the worms. However, four of these compounds stimulated glucose consumption by *Onchobothrium pseudo-uncinatum*, and one stimulated glucose uptake by *Calliobothrium verticillatum*. Glucose consumption by *C. verticillatum* and *L. tenuis* was not affected by quercetin, *p,p'*-biphenol, bis(*p*-hydroxyphenyl)methane, and diethylstilbesterol.

The worms contained at least 3 carbohydrate fractions, 2 of which were stable to alkaline digestion. *L. tenuis*, *O. pseudo-uncinatum*, and *Inermiphyllidium pulvinatum* synthesized a net increase in polysaccharide during four hours incubation in the presence of glucose.

A substance that was presumably lactic acid was excreted by all 8 species of cestodes.

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SHORT COMMUNICATION

TISSUE RESPIRATION AND GLYCOGENOLYSIS IN BIRDS AND YOUNG MAMMALS

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Abstract—1. Adult pigeons and young rabbits housed at 32°C were exposed to 48° and 45° respectively for 2½ h. In the young rabbits the Q_{O_2} of succinate in the liver was increased but there was no increase in Q_{O_2} value of pyruvate or ascorbate in liver or kidney.

2. In pigeons there was an overall increase of Q_{O_2} to succinate, pyruvate and ascorbate substrates.

3. Glycogenolysis was increased by 200 per cent in pigeon liver slices; there was no increase in the rabbit liver slices.

EARLIER experiments on the effect of temperature changes on the tissue respiration of vertebrates (Sadhu, 1959; Chaudhuri, Anubha & Sadhu, 1960; Chaudhuri & Sadhu, 1961) showed that there was a significant difference in the responses of poikilotherms to temperature changes, as measured by their tissue respiration. More recently we have found that exposure of pigeons to ambient temperatures of 48°C, as compared to controls at 32°C, increased the plasma bound iodides (PBI) to 143 per cent, whilst in young rabbits, exposure to 45° instead of 32° increased the plasma bound iodides to 124 per cent. This increase could have been due to the non-utilization of the iodides by the tissues in the hot environment and could reflect a difference in the tissue response of the pigeon and rabbit to a temperature increase. We therefore decided to study the tissue respiration of kidney and liver slices of pigeons and rabbits that had been exposed for a short time to a higher temperature, to see if this differed from that of normal animals. Twenty young albino rabbits weighing about 300 g and twenty adult pigeons weighing about 270 g were used. Half the animals in each group were exposed to a temperature of 45° (rabbits) and 48° (pigeons). The temperature was kept constant (±0·03°C) and the animals were kept there for 2½ h. The animals kept as controls were maintained at 32°C. The animals were then killed by a blow on the head and their tissues (liver and kidneys) removed, sliced and examined experimentally.

The results are shown in Tables 1 and 2. The heat-exposed rabbit liver showed an increase in succinate oxidation whilst the pyruvate and ascorbate oxidations

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were depressed. In the kidney slices there was no increase in oxidation. In the pigeon, heat exposure caused a general increase in oxidation by the kidney slices to all three substrates. Both the pigeon and the rabbit showed a depletion of liver

TABLE 1—TISSUE RESPIRATION STUDIES IN LIVER AND KIDNEY SLICES OF PIGEON AND YOUNG RABBITS HOUSED AT 32°C AND THOSE EXPOSED TO HIGHER AMBIENT TEMPERATURE OF 48°C FOR PIGEONS AND 45°C FOR YOUNG RABBITS AS Q_{O_2} VALUE WITH SUBSTRATE SUCCINATE, PYRUVATE AND ASCORBATE OVER THE VALUE WITHOUT SUBSTRATE. Q_{O_2} = mm^3 OXYGEN CONSUMED/mg DRY WT. OF TISSUE/HR. TEN ANIMALS IN EACH GROUP

Species	Liver slices			Kidney slices		
	Succinate	Pyruvate	Ascorbate	Succinate	Pyruvate	Ascorbate
Rabbit 32°C	15·2 ± 0·6	1·4 ± 0·05	2·7 ± 0·1	18·3 ± 1·3	3·0 ± 0·05	2·7 ± 0·02
45°C	20·2 ± 1·1	0·5 ± 0·02	1·1 ± 0·02	17·9 ± 1·2	2·0 ± 0·03	1·2 ± 0·01
Pigeon 32°C	10·4 ± 0·9	1·6 ± 0·03	0·6 ± 0·02	13·1 ± 1·2	1·2 ± 0·04	2·5 ± 0·03
48°C	15·9 ± 1·2	2·2 ± 0·04	0·7 ± 0·03	16·3 ± 1·1	3·0 ± 0·02	3·9 ± 0·04

TABLE 2—STUDY OF GLYCOGENOLYSIS IN LIVER SLICES OF PIGEON AND BABY RABBIT EXPOSED TO WARM ENVIRONMENT

Group	Initial glycogen mg/100 mg	Glucose liberation mg/100 mg liver			Glucose liberation mg in 40 min (c − a) in mg	Glucose liberation mg per mg initial glycogen in 40 min (c − a) in mg
		20 min (a)	40 min (b)	60 min (c)		
Pigeon at 32°C	1·12 ± 0·20	0·42	0·50	0·58	0·16 ± 0·02	0·15 ± 0·02
Pigeon at 48°C	0·31 ± 0·04	0·32	0·36	0·41	0·09 ± 0·01	0·28 ± 0·04
Rabbit at 32°C	1·50 ± 0·12	0·49	0·60	0·60	0·20 ± 0·05	0·14 ± 0·01
Rabbit at 45°C	0·56 ± 0·03	0·34	0·37	0·41	0·07 ± 0·01	0·13 ± 0·02

glycogen following heat treatment. In the rabbits there was no significant difference in glucose liberation but in the pigeon, the heat-treated animals showed an increase in the rate of glycogenolysis.

It is suggested that the tissue respiration in the heat-treated animals agrees with our findings on PBI serum levels in the pigeon and the young rabbit following heat treatment. On heat treatment the requirements of the tissues for thyroid hormones would most likely be reduced, and the level of the serum PBI would increase due to non-utilization. This would explain the increased PBI levels in the pigeon compared to the rabbit; the pigeon also having the greater increase of respiration following heat treatment.

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SHORT COMMUNICATION

REACTIONS CARDIAQUES ET VARIATIONS DE MASSE SANGUINE CHEZ LES TELEOSTEENS

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Abstract—1. Injection of 1 ml of saline into the hepatic vein of the catfish *Ameiurus nebulosus* brings about a marked tachycardia.

2. This tachycardia can be prevented to a large extent by the prior injection of atropine, or the cutting of both vagi.

3. Various interpretations of the tachycardia are discussed, and it is suggested that the tachycardia following an increase of blood volume may be due to a direct action of pressure on the myocardium. This direct action can be modified by the vagus.

INTRODUCTION

DANS le cadre d'études physio-écologiques, Serfaty et Labat (1960) ont pu mettre en évidence des réactions cardiaques significatives chez les Poissons au cours de modifications expérimentales de la salinité du milieu. Selon une hypothèse émise par ces auteurs, la tachycardie observée, dans certains cas, serait liée à une augmentation possible de la masse sanguine.

Le but du présent travail est de déterminer la valeur de cette hypothèse en faisant appel à des mécanismes étudiés chez d'autres espèces. Il est connu depuis longtemps que, de façon générale, la pléthore sanguine veineuse entraîne une élévation de la fréquence et du débit cardiaque.

Bainbridge (1915) eut, le premier, le mérite d'attirer l'attention sur le déterminisme de ce mécanisme; il attribua, en effet, à une inhibition de l'action vagale, l'accélération cardiaque observée au cours des hypertensions veineuses.

Les travaux effectués depuis n'ont pas permis de généraliser l'explication fournie par Bainbridge. D'autre part, Sassa et Miayazaki (1920), chez le Lapin et le Chat, n'ont pu obtenir le réflexe cardio-accélérateur. Selon De Graff et Sands (1925), 50 pour cent des Chiens seulement réagissent à l'hypertension veineuse. Ballin et Katz (1941) font la discrimination entre Chien non anesthésiés et Chiens anesthésiés; les premiers répondent régulièrement par une cardio-accélération à la perfusion veineuse, les derniers réagissent beaucoup plus irrégulièrement.

Fergusson, Shadle et Gregg (1953) montrent que seules les perfusions rapides sont efficaces, les résultats obtenus avec des perfusions lentes étant très variables.

Coleridge et Linden (1955) remarquent que sur dix Chiens, trois seulement présentent le réflexe sans équivoque, les sept autres qui ne présentent pas d'accélération significative ont avant l'expérience une fréquence très élevée.

Nous avons montré chez les Téléostéens marins, en particulier, *Mugil capito* C. et V., *Mugil auratus* R. et *Cantharus cantharus* C. et V., que le passage en eau douce déclenche une tachycardie d'autant plus rapide dans le temps que le sujet est moins euryhalin. Pour expliquer ce phénomène Serfaty et Labat (1960) ont suggéré l'existence d'un mécanisme peut être identique à celui de Bainbridge et Mac-Dowal. Mais, dans ces expériences, rien ne permettait de montrer de façon certaine que la masse sanguine était accrue. Ces expériences ont été reprises sur la Carpe, chez laquelle un bain d'eau salée à la concentration de 15‰ déclenche d'après Fontaine et Callamand (1946) une augmentation de la masse sanguine après 36–48 h d'immersion. En effet, Labat et Serfaty (1961) ont observé chez de tels sujets placés dans des conditions identiques, une tachycardie légère mais significative apparaissant après 36–48 h d'immersion. Le parallèlisme entre ces deux phénomènes semblait appuyer notre première hypothèse. D'autre part, Jensen (1961) a montré que le myocarde d'*Eptatretus stoutii** possédait un "pace-maker" myocardial, diffus et sensible à la pression.

C'est alors que nous nous sommes demandés ce que pouvait produire une injection de sérum physiologique sur la fréquence cardiaque d'un Téléostéen d'eau douce. Soulignons que le cœur des Poissons est essentiellement veineux et qu'il est pratiquement sous le seul contrôle vagal.

TECHNIQUE

Après un certain nombre de tâtonnements sur divers Téléostéens: Carpes (*Cyprinus carpio* L.), Tanches (*Tinca tinca* L.), Perches (*Perca fluviatilis* L.), nous avons choisi le Poisson-Chat (*Ameiurus nebulosus* R.), dont l'anatomie se prête bien à cette expérimentation.

Nous avons opéré sur une trentaine de sujets maintenus le ventre en l'air dans un aquarium (température 11–12°), région cardiaque intacte, seule la région postérieure du septum était ouverte pour permettre la découverte de la veine sus-hépatique. La partie antérieure du Poisson baignait dans l'eau afin de permettre une respiration normale. Des injections de 1 cm^3 de sérum physiologique† étaient effectuées en 15 ou 20 sec en moyenne dans la veine sus-hépatique, ce qui revient à augmenter, la masse sanguine de 1/7ème environ dans le cas du Poisson-Chat. Le flacon de sérum était maintenu à la température de l'eau de l'aquarium.

* Cœur branchial énervé de Myxine de Californie.

† Formule du sérum physiologique utilisé:

Sol. 1.	$NaCl$ anhydre RP	: 0,136 M.	70 parties
	KCl anhydre RP	: 0,002 M.	
	Cl_2Ca anhydre RP	: 0,001 M.	
	Na_2HPO_4, $12H_2O$ RP	: $0{,}00005 = 5 \times 10^{-5}$ M.	
Tampon phosphate:			
	Na_2HPO_4, $12H_2O$ RP	: 0,021 M.	15 parties
	KH_2PO_4 anhydre	: 0,160 M.	1 partie
Glucose anhydre pur		: 0,3 M.	5 parties

Les enregistrements électrocardiographiques ont été effectués selon la technique décrite par Serfaty et Raynaud (1956).

RESULTATS ET DISCUSSION

(1) L'injection rapide de 1 cm^3 de sérum physiologique dans la veine sus-hépatique entraîne une tachycardie significative de l'ordre de 25–50 pour cent (Fig. 1).

On ne peut donc que constater la relation qui existe entre cette tachycardie et la pléthore sanguine créée par l'injection de 1 cm^3 de sérum physiologique dans le système veineux précardiaque. Cette relation nous semble d'autant plus valable que nous avons éliminé l'interférence que pouvait produire une tachycardie traumatique consécutive à l'implantation d'une aiguille. Nous avons attendu que cette tachycardie disparaisse avant de faire l'injection.

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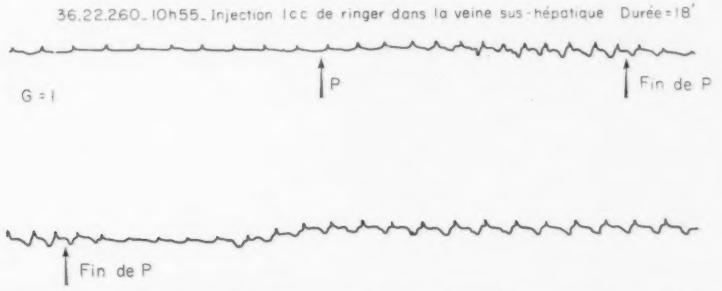

FIG. 1. Tracé électrocardiographique montrant une tachycardie provoquée par l'injection de 1 cm^3 de sérum physiologique dans la veine sus-hépatique. P: début de l'injection. Fin de P: fin de l'injection (durée 18 secondes).
Le rythme cardiaque normal est rétabli peu après la fin de l'injection.
Exp. 36, 22 Février 1961. *A. nebulosus*—Poids: 160 g—t°: 12°C.

(2) Par suite d'une tachycardie consécutive à la section vagale ou à l'atropinisation (Fig. 2), la réponse cardiaque est fortement atténuée après l'injection. Notons que la vagotomie produit souvent une forte bradycardie très fugace due à une excitation mécanique par traumatisme.

(3) La section vagale ou l'atropinisation diminue certes très fortement la réponse cardiaque à l'injection, mais laisse néanmoins apparaître une légère tachycardie supplémentaire. D'une manière plus générale, ceci tendrait à montrer que si la section vagale élève fortement la fréquence du cœur, elle ne confère cependant pas obligatoirement une fréquence maximum à ce cœur; des facteurs positifs comme la température pouvant agir directement sur le myocarde; c'est ainsi qu'une augmentation de température détermine une tachycardie même chez les sujets bivagotomisés (Fig. 3).

Autrement dit, une tachycardie initiale peut masquer, en partie, l'effet cardiaque dû à une élévation de la masse sanguine. D'ailleurs, nos résultats confirment ceux obtenus par la plupart des auteurs dans leurs expérimentations sur les Mammifères.

Pour expliquer le mécanisme de tachycardie consécutive à la pléthore sanguine, trois hypothèses sont possibles:

Ou bien l'élévation de la masse sanguine exerce une activité cardio-accélératrice par inhibition de l'action vagale, dans ce cas la double vagotomie devrait faire disparaître l'effet résultant de la pléthore sanguine.

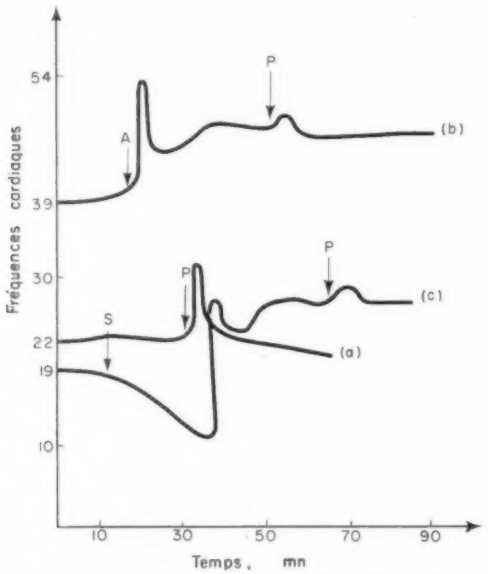

FIG. 2. Le tracé (a) montre nettement la tachycardie obtenue après injection de 1 cm³ de sérum physiologique (P), le rythme normal se rétablissant 3 min après l'injection.

Exp. 25, 27 Janvier 1961. *A. nebulosus*—Poids: 250 g—t°: 9°C.

Le tracé (b) montre qu'après atropinisation (A), s'installe une tachycardie persistante due à la suppression du frein vagal. Ici, l'injection de 1 cm³ de sérum physiologique (P) ne provoque qu'une très légère augmentation peu significative du rythme cardiaque.

Exp. 33, 13 Février 1961. *A. nebulosus*—Poids: 160 g—t°: 11°C.

Le tracé (c) montre qu'après la section bilatérale des branches cardiaques du vague (S), s'installe une tachycardie persistante due à la suppression du frein vagal. Ici encore l'injection de 1 cm³ de sérum physiologique (P) n'entraîne qu'une modification peu significative du rythme cardiaque.

Exp. 34, 15 Février 1961. *A. nebulosus*—Poids: 180 g—t°: 11°C.

Ou bien l'augmentation de la masse sanguine aurait un effet sur le myocarde lui-même à savoir le tissu cardiaque et le système nerveux intra-cardiaque, représenté par les voies de transmissions auriculo-ventriculaires (Ripplinger, 1950).

Une troisième hypothèse mixte faisant appel à une association des deux premières.

Il est permis d'accepter, surtout chez les Poissons, sujets très vagotoniques, une telle suggestion. La réponse à l'effet pléthoro-sanguin étant plus nette chez les sujets intacts que chez les sujets bi-vagotomisés.

Pour résumer cette interprétation de la tachycardie consécutive à l'élévation de la masse sanguine chez le Poisson, nous proposons le mécanisme suivant.

Comme chez les Mammifères le rythme cardiaque normal du Poisson résulte surtout de l'action freinatrice des vagues. La pléthore sanguine entraîne une

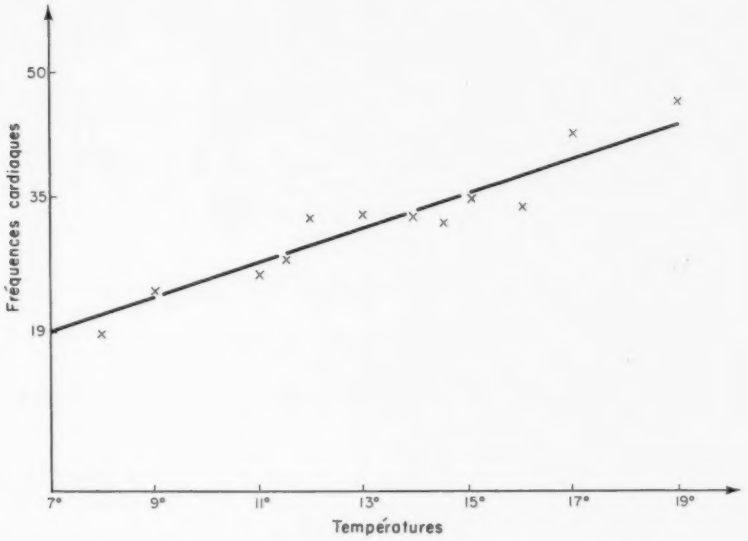

FIG. 3. La fréquence cardiaque augmente avec la température chez le Poisson-Chat bivagotomisé.
Exp. 44, 25 Mars 1961. *A. nebulosus*—Poids: 210 g.

élévation de la fréquence. Sur un cœur privé de son innervation vagale, cette tachycardie est moins marquée car elle s'exerce sur un cœur ayant un rythme élevé du fait de la section des fibres cardio-modératrices. Mais cette légère tachycardie présente néanmoins un grand intérêt car elle semble montrer que l'élévation de la masse sanguine agit au moins en partie sur le myocarde lui-même; il n'est donc pas exclu que la réaction cardio-accélératrice résulte pour une part, d'une action positive de la pléthore sanguine sur le myocarde, réaction que l'effet vagal serait incapable de contenir.

Une augmentation de la masse sanguine détermine chez le Poisson-Chat une tachycardie qui s'estompe rapidement. Pour expliquer cette tachycardie, il semble qu'il faille faire appel, peut-être à un réflexe vagal, mais aussi à une réaction intra-cardiaque.

L'hypothèse qui a été suggérée pour expliquer l'effet chronotrope positif chez les Téléostéens marins ou d'eau douce, lors des variations de salinité dans les conditions mentionnées ci-dessus, trouverait un premier argument valable dans le mécanisme de ces expériences.

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STRUCTURE AND FUNCTION OF SUBCELLULAR PARTICLES

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1. INTRODUCTION

IT is traditional for the lectures given at the plenary sessions of the triennial International Congress to deal with a broad area of biochemical investigation which currently is the focus of intense research effort. Those of us in all parts of the world who are engaged in the study of subcellular structure and function are naturally very pleased and feel honored that the organizers of the Moscow International Congress of 1961 have selected this topic as the subject matter of one of the lectures of the present plenary session. In that context it is appropriate to point out that our hosts, the biochemists of the Union of Soviet Socialist Republics, have made many outstanding contributions to our knowledge of subcellular structure and function. Certainly the names of Belitzer, Engelhardt and Sissakian are assured a place of honor among the investigators in this field.

It is only within the past decade that biochemists have seriously come to grips with the problems of subcellular structure and function. There were at first formidable methodological and conceptual problems to be overcome before a systematic experimental attack could be undertaken. I have decided not to review on this occasion the various stages in the historical development of the field. As some of you know full well, progress at first was painful and slow. Many of the ideas and concepts which are generally accepted today were at one time the targets of severe criticism and extended controversies. But we shall pass over the historical developments and address ourselves to each of five propositions which go to the heart of current investigations: (1) that the energy transformations of living systems are carried out by structured, subcellular devices which I shall refer to as biochemical machines; (2) that some general principles underlie the performance of all biochemical machines; (3) that the structure of at least one biochemical machine, viz., the mitochondrion, can now be described in chemical terms and in considerable detail; (4) that the design of some of these machines has applicability to all forms of living systems; and (5) that the function of these machines is inextricably tied in with their structure.

We may classify structured subcellular systems into three broad functional groups: (1) transducing machines such as the mitochondrion or the chloroplast, which catalyze energy transformations; (2) replicating machines such as the ribosomes which catalyze the replication of proteins or other macromolecules; and (3) integrated metabolic systems such as the particles involved in the synthesis of fatty acids and cholesterol which catalyze multi-enzyme synthetic processes.

Because of the limitation of time I shall deal with only the first group of subcellular structures, viz., the transducing machines (cf. Table 1). These are all subcellular

TABLE 1—ENERGY TRANSFORMATIONS IN LIVING CELLS

Transformation	Organ
Chemical to electrical energy	Brain, nerve, receptor elements in
Sound to electrical energy	Nose and tongue, inner ear
Light to electrical energy	Retinal rods and cones
Chemical to osmotic energy	Tubular elements of the kidney
Light to chemical energy	Chloroplast
Chemical to light energy	Luminescent organ of firefly
Chemical to mechanical energy	Muscle
Hydrostatic to electrical energy	Neural elements in the inner ear

structured devices built up of a large number of identical repeating units which are arranged in series. The repeating units of each of the biochemical machines contain the unique molecules that carry out the transduction (cf. Table 2). Trans-

TABLE 2—MOLECULAR INSTRUMENTS OF ENERGY TRANSFORMATIONS

Oxidative Phosphorylation	*Photosynthesis*
DPN	chlorophyll
coenzyme-Q	cytochrome
cytochrome-c	
Muscular Contraction	*Luminescent Organ*
myosin	flavin
actin	adenosinemonophosphate
Retina	
rhodopsin	
(retinene, opsin)	

ductions brought about by biochemical machines are implemented at the molecular level. That is to say, the transducing molecules accept energy in one form and convert this energy to another form. The activity of the transducing molecules requires special conditions, and the structure of the machine has to be described in terms of a chemical architecture designed, so to speak, to satisfy the requirements for the efficient performance of the transducing molecules.

2. THE MITOCHONDRION AS A PROTOTYPE OF TRANSDUCING MACHINES

Since so much of the present lecture will be devoted to the mitochondrion, it is appropriate to inquire to what extent some of the principles which have been found to apply to the mitochondrion can be extended to other biochemical transducing machines. Information on this point is still fragmentary but there are several straws in the wind. Electron microscopic evidence has borne out the close structural

similarities between all the transducing machines yet examined (e.g. chloroplasts, mitochondria and receptor elements in the retinal rods and cones) (Fernandez-Moran, 1959; Sjöstrand, 1960). The properties of multiple repeating units, of double membrane particulate structure and of high lipide content apply wherever they have been looked for. At first glance it may be difficult to see why biochemical machines with different transducing functions should have anything in common. The specialized molecules and the functional apparatus involved in transduction are obviously unique to each machine. But it is more than likely that the structural characteristics of biochemical transducing machines are very similar indeed. It is as though an important architectural principle were common to them all. If we think of the transducing molecules as attached to a structural framework and look upon this framework as the constant element in different types of biochemical machines, then it would not be so strange after all that these machines should show strong resemblances at the electron microscopic level. Later on we shall discuss the structural protein of mitochondria which may well be one of the elements of universality in biochemical transducing systems.

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3. FUNCTIONS OF THE MITOCHONDRION

The primary function of all mitochondria is to couple the synthesis of ATP (from ADP and inorganic phosphate) to the aerobic oxidation of some metabolite. The coupling of ATP synthesis to oxidation is the constant; the variable is the nature of the substance oxidized. In most cases the intermediates of the citric acid cycle are the principal if not the only substances which undergo oxidation. But this is not always true. α-Glycerophosphate (Sacktor *et al.*, 1957; Bucher *et al.*, 1958) and β-hydroxybutyrate (Lehninger, 1955) in some mitochondria can replace citric cycle substrates completely. In bacteria even inorganic substances can serve as sources of electrons (Stephenson *et al.*, 1931; Aleem *et al.*, 1960). Thus the origin of the electrons is secondary; the primary consideration is the coupling of this electron flow to a synthetic process (the synthesis of ATP). The transduction in this case is the conversion of chemical energy from the bond energies of certain metabolites to the bond energies of ATP. This is achieved by oxidation of the metabolites by oxygen, and by conservation of the energy thus released for the synthesis of ATP.

There are, generally speaking, three parameters of the primary mitochondrial function: (1) citric cycle oxidations; (2) electron transport; and (3) oxidative phosphorylation. The last two are the constants while the first is a variable for mitochondria generally. In most mitochondria which carry out the citric cycle, a variety of substances other than the actual intermediates of the cycle can be oxidized as well (cf. Table 3). These may be substances such as amino acids (Taggart *et al.*, 1949) or fatty acids (Grafflin *et al.*, 1946) which give rise eventually to one or another intermediate of the citric acid cycle or they may be substances such as α-glycerophosphate (Sacktor *et al.*, 1957; Bucher *et al.*, 1958), β-hydroxybutyrate (Lehninger, 1955) and choline (Rendina, 1959), which undergo only a single oxidative change unrelated to and unaffected by the citric acid cycle.

Mitochondria generally manifest enzymatic activities additional to that of the primary coupling function. These include processes as diverse as synthesis of hippuric acid from glycine and benzoyl coenzyme A (Sarkar, 1952), synthesis of

TABLE 3—OXIDIZABLE SUBSTRATES OF MITOCHONDRIA OTHER THAN CITRIC CYCLE COMPONENTS

Substrate	Oxidation Product
β-Hydroxybutyrate	Acetoacetate
L-α-glycerophosphate	Dihydroxyacetonephosphate
Fatty acyl CoA esters	α-β unsaturated acyl CoA esters
β-Hydroxyacyl CoA esters	β-Ketoacyl CoA esters
D-aspartate	Oxalacetate
L-proline	Pyrroline carboxylic acid
Choline	Betaine aldehyde

phospholipide (Kennedy, 1954, 1956) by interaction of cytidine diphosphocholine or cytidine diphosphoethanolamine with a diglyceride, and synthesis of cytochrome-*c* from amino acids (Kalf *et al.*, 1959). The processes which are not directly related to the primary coupling phenomena appear not to be localized in the structured elements which carry out the transduction process. As we shall see later the mitochondrion is a composite of structured elements and spaces. On the basis of available evidence, the systems implicated in these secondary activities can be assigned to the internal spaces which surround the structured elements of the mitochondrion, and while geographically speaking these systems are intramitochondrial, they are from a structural standpoint extramitochondrial. The internal spaces between the structured elements of the mitochondrion are essentially extensions of the cytoplasm. Within these internal spaces specific cytoplasmic components become trapped.

It cannot be emphasized too strongly that the mitochondrion is a complete, operational unit in the sense that it contains all the coenzymes, cofactors and enzymes required to carry out its functions (Green, 1951). No additions to the isolated mitochondrion other than inorganic phosphate, molecular oxygen and inorganic ions such as Mg^{2+} are necessary. The idea that enzymes essential for mitochondrial activity may be located outside the mitochondrion has been completely discredited.

4. STRUCTURE OF THE MITOCHONDRION

The tool of electron microscopy has been invaluable in revealing the fine structure of the mitochondrion. The studies of Fernandez-Moran (1959), Palade (1956), Ris (Ziegler *et al.*, 1958), Sjöstrand (1960) and others have led to the generalized picture of the mitochondrion shown in Fig. 1. The essential elements are (1) a three-banded membrane structure which surrounds the mitochondrion (external system of membranes) and (2) a series of three-banded membrane

structures which are contained in the interior of the mitochondrion (internal system of membranes or cristae or septa). The three-banded structure consists of twin electron-dense bands separated by an electron-transparent band of uniform

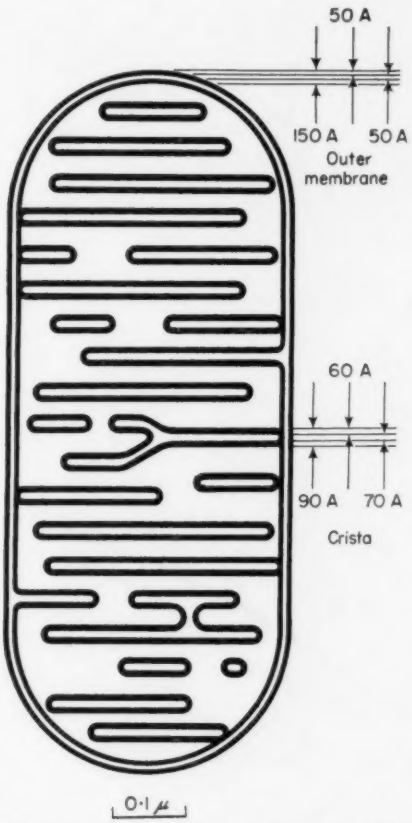

FIG. 1. Generalized diagrammatic representation of a rod-shaped mitochondrion.

width. To avoid confusion we shall refer to all three bands as the membrane and this will still apply whether or not the membrane can be resolved into even more bands. A three-dimensional representation modelled after Anderssen-Cedergren (1959) of the membrane components of a mitochondrion is shown in Fig. 2. The membrane elements (external and crista) which are the structured areas of the mitochondrion do not account for the entire volume. The intercristate spaces of mitochondria from some sources are relatively extensive whereas these spaces in mitochondria from other sources are limited in extent. The number of cristae is inversely related to the number of ancillary functions. Heart mitochondria which have few ancillary functions are packed with cristae (cf. Fig. 3), whereas liver mitochondria which lead in the number of ancillary functions have relatively

few cristae. This correlation suggests that the space between cristae may well be the locale for secondary mitochondrial functions such as the synthesis of protein, phospholipide and fatty acid.

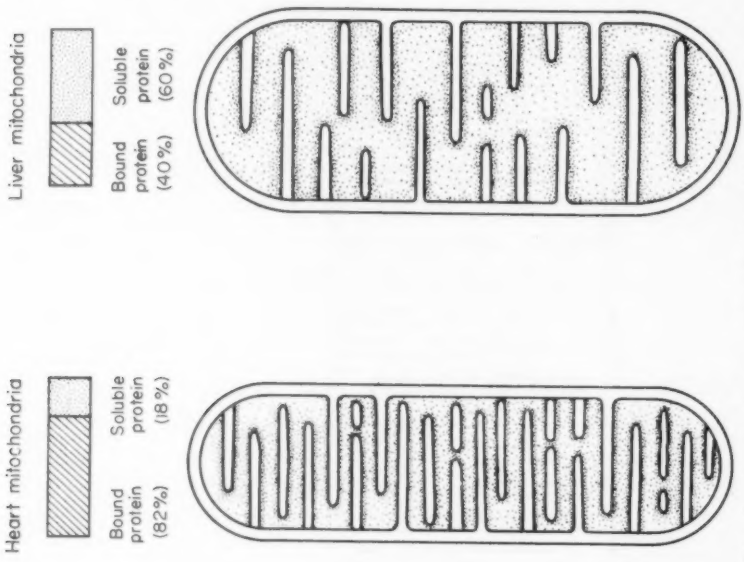

FIG. 3. Comparison of the mitochondrion of heart and liver with respect to number of cristae and the ratio of particle-bound protein to soluble protein.

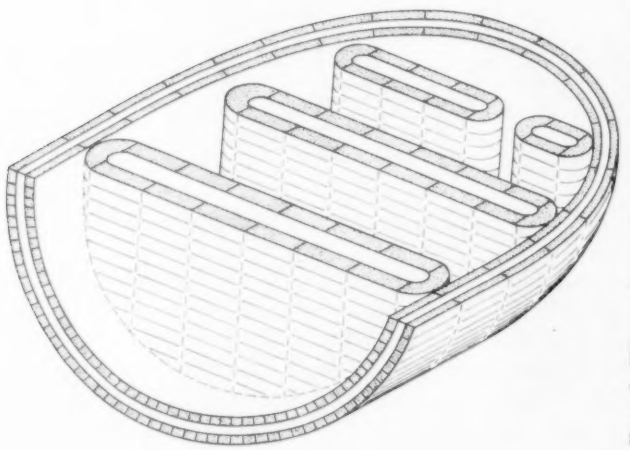

FIG. 2. Three-dimensional representation of a mitochondrion showing the stacking of the units of mitochondrial function in the crista.

The chemical studies of the mitochondrion have led to the following conclusions about the smallest common denominator of mitochondrial activity. The unit must be an asymmetric, probably rod-like supermolecule (in the sense that would apply to a virus particle) for molecular weight about 2×10^6 (Green *et al.*, 1961).

This unit should contain about 30 per cent of lipid and over 50 per cent of a structural protein which is not concerned in oxidoreductions.

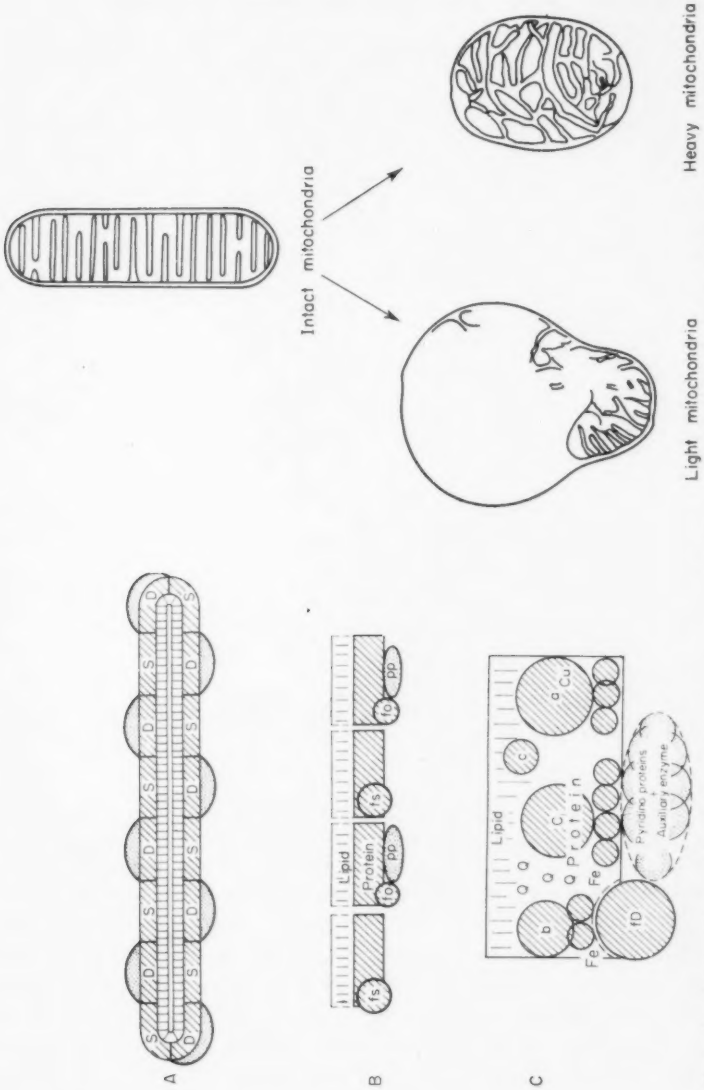

FIG. 4. Association of pyridinoprotein enzymes and other auxiliary enzymes of the citric acid cycle with the electron transport unit. The association shown diagrammatically for a single unit (diagram C) is extrapolated to the crista (diagrams A and B). The symbols S and D (in diagram A) refer to the succinic and DPNH chains respectively.

FIG. 5. Conversion of mitochondria to light and heavy mitochondria. In the light mitochondria the cristae separate from the external membrane and concentrate in a pocket at one end. In the heavy mitochondria the cristae are vesiculated and tightly packed. (After Ziegler *et al.* (1958).)

It is important to ascertain whether the functional unit can be accommodated within the structured membranes of the mitochondrion as revealed by the electron microscope (Oda, 1958). As shown in Fig. 2 the dimensions of the external membrane and of the cristae would permit nicely a system of stacked units which

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fulfil the biochemical requirements. The units may be stacked in other ways than that shown in Fig. 2 and there are other ways in which the individual unit could be arranged. At present one cannot decide between various alternatives.

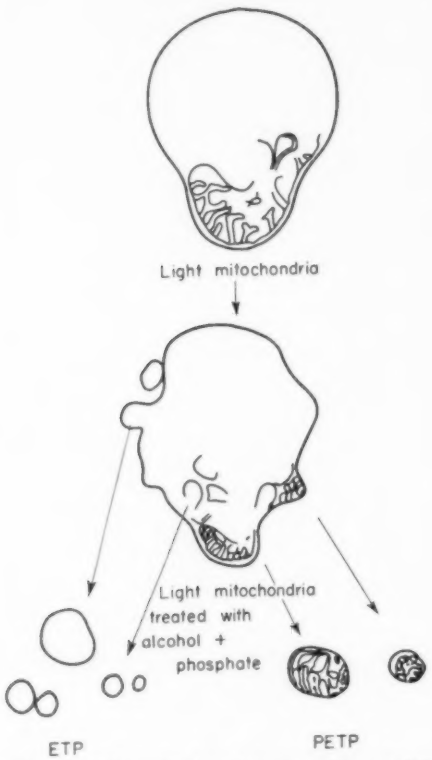

FIG. 6. The conversion of light mitochondria to ETP and PETP. The vesiculation of the external membrane leads to the formation of ETP while the disengagement of small packets of cristae lead to the formation of PETP (miniature mitochondria with phosphorylating properties). (After Ziegler *et al.* (1958).)

Associated with the electron transport chain of the functional unit is a set of auxiliary enzymes and cofactors which are involved in implementing (1) the citric acid cycle, (2) fatty acid oxidation, and (3) the conversion of compounds which give rise to citric cycle intermediates (Green *et al.*, 1948). These enzymes are intimately associated with the functional unit. There are several lines of evidence which suggest that these auxiliary enzymes are arranged in organized complexes and in constant molecular proportions. Each functional unit may have associated with it a fixed complement of these auxiliary enzymes and cofactors (cf. Fig. 4). When the mitochondrion is disintegrated by sonic vibrators, most of these auxiliary enzymes and cofactors become detached from the structured electron transport

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system and the capacity for citric and fatty acid cycle oxidations is lost (Linnane *et al.*, 1958). Clearly the bonds between the structured part of the electron transport chain and the auxiliary components are not strong. With disintegration of the mitochondrion the repeating units remain in the form of submitochondrial particles while the detached enzymes become soluble.

It would be expected that in the process of converting a mitochondrion to smaller fragments the mitochondrion would fall apart into its component units. The electron microscope evidence clearly suggests that such is the case (Ziegler *et al.*, 1958) (cf. Figs. 5 and 6). The cristae can be separated from the external membrane and in turn both of these structured elements can be comminuted into yet smaller units which approach in dimensions particles of molecular weight about 2×10^6 (Chen *et al.*, in preparation) and these units tend to round up in the form of vesicles such as are characteristic of ETP.

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5. THE QUESTION OF BACTERIAL MITOCHONDRIA

On the basis of the evidence of chemical composition and enzymatic properties, the repeating unit of the respiratory particles of bacteria such as Azotobacter may be considered to be for all practical purposes the same as that of the heart mitochondrion (Bruemmer *et al.*, 1957). The purified bacterial particles have much the same composition and act in precisely the same way as mitochondria, i.e. they couple the oxidation of citric cycle substrates by oxygen to the synthesis of ATP (Tissieres *et al.*, 1955) (cf. Table 4). From a functional point of view it is an

TABLE 4—COMPARISON OF THE ELECTRON TRANSPORT PARTICLE OF BEEF HEART MUSCLE AND AZOTOBACTER

	Beef heart	Azotobacter
Activities		
(μM substrate/min/mg prot. at 38°)		
Succinic-oxygen	2·0	2·4
DPNH-oxygen	5·7	6·8
Succinic-ferricyanide	5·0	3·5
Succinic-cytochrome-*c*	0·1	0·3
DPNH-cytochrome-*c*	0·2	0·2
Composition		
Hemes	a, b, c_1, c	a_2, b_1, c_4, c_5
Lipid	30%	29%
Flavin : heme : Fe : Cu (molecular or atomic proportions)	1/5/14/3·7	1/5/16/2

Data taken from Bruemmer *et al.* (1957), Doeg and Ziegler (unpublished).

academic question whether the respiratory particles of Azotobacter or of other aerobic bacteria should be called mitochondria. It is true that these particles are

smaller and that their origin or locale in the cell is different, but they do show the membrane structure characteristic of mitochondria even though this structure does not have the repetitive and regular pattern of classical mitochondria (Ris *et al.*, unpublished). From a functional standpoint it is immaterial whether the repeating units of the bacterial electron transport system are arranged in the form of individual cristae rather than in a system of cristae or whether they are closely associated with other structured elements of the bacterial cell. The reluctance to consider bacterial respiratory particles as mitochondrion-like particles has had the effect of obscuring the universality of the chemical blueprint which underlies the mitochondrial machine.

6. STRATEGY FOR STUDYING MITOCHONDRIAL STRUCTURE AND FUNCTION

There are obviously two diametrically opposite ways of tackling the problem of mitochondrial structure and function. The first is to preserve the mitochondrion and to study the performance of the intact structure. The second is to tear the mitochondrion apart and deduce the properties of the whole from the properties of the derivative fragments. The first approach is that of classical physiology; the second is that of the latter-day enzymologist. There is of course a limit to the information which can be acquired by studying the intact mitochondrion. Physical tools like the spectrophotometer can assist the probing process, but these tools even when used with great ingenuity can rarely match the power of the direct method. It is of interest to point out that not a single new component of the electron transport chain besides those described by Keilin some 30 years ago was recognized by the direct study of the mitochondrion. On the other side of the ledger, the spectrophotometric studies of Chance and his school (Chance *et al.*, 1956) have extended enormously our fund of information about the relative rates of oxidation and reduction of the various cytochromes in mitochondria, and this information has been invaluable as a guide to the study of submitochondrial particles. The combination of the two approaches has led to spectacular developments in the last few years.

7. LARGE-SCALE ISOLATION OF MITOCHONDRIA

In attempting to resolve the mitochondrion into simpler units it was necessary to isolate mitochondria on a large scale and to find conditions for stabilizing mitochondrial activity. The discovery that the mitochondrion of heart muscle was highly stable and could be stored indefinitely in the frozen state encouraged us in 1954 to develop the technique of the large-scale isolation of mitochondria from beef heart muscle collected from the slaughter house (Hele, 1954). By now we have produced over 200 kg dry weight of heart mitochondria in our mitochondrial factory, located in the basement of our institute. The large-scale isolation of mitochondria also made it necessary to develop the technique for the fractionation of submitochondrial particles on an extended scale. We were fortunate that the Spinco ultracentrifuge—an instrument which was ideally suited for this purpose—was commercially available at the time.

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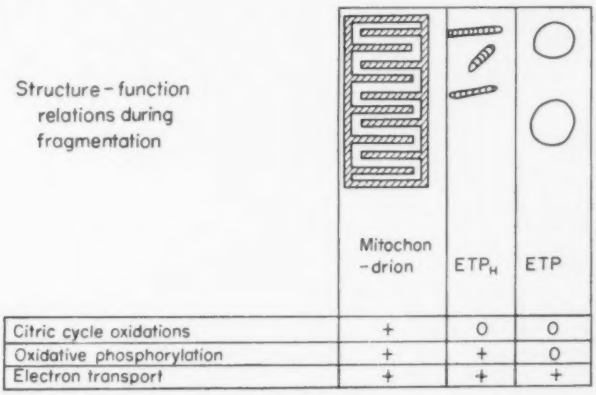
8. STEPWISE SIMPLIFICATION OF MITOCHONDRIAL STRUCTURE AND FUNCTION

The intact mitochondrion, as mentioned earlier, couples the oxidations of the citric acid cycle to the synthesis of ATP. When the mitochondrion is fragmented, however, its capacity to carry out the complete citric acid cycle is lost. Exposure of the mitochondrion to short periods of sonic oscillation yields a particle, known as ETP_H, which is capable of coupling the oxidation of DPNH or succinate to the synthesis of ATP (Linnane *et al.*, 1958). ETP_H can, in turn, be converted to an even simpler particle, known as ETP, which is still able to catalyze the oxidation of DPNH or succinate by molecular oxygen, but which is no longer able to couple these oxidations to the synthesis of ATP (Linnane & Ziegler, 1958; Green *et al.*, 1957) (cf. Fig. 7). Thus fragmentation of the mitochondrion into submitochondrial



Citric cycle oxidations	+	0	0
Oxidative phosphorylation	+	+	0
Electron transport	+	+	+

FIG. 7. Structure–function interrelationships of mitochondria, ETP_H and ETP.

particles leads to the stepwise elimination of the original functional capacities. Attendant upon this serial loss of catalytic activities is a corresponding simplification of structure. ETP_H no longer shows the organized array of crista typical of mitochondria while ETP no longer shows the thickened three-banded membranes characteristic of ETP_H (Ziegler *et al.*, 1958). The organized array of cristae is the structural prerequisite for carrying out the citric acid cycle while the thickened three-banded membrane is one of the structural prerequisites for preserving coupling function. The simplification process can be extended even farther but these extensions will be considered at a later point in the talk.

9. SIMPLIFICATION OF THE ELECTRON TRANSPORT CHAIN

The electron transport chain of the mitochondrion may be looked upon as a composite of two main segments. The first is concerned in the transfer of electrons from substrate to DPN while the second is concerned in the transfer of electrons from DPNH or succinate to molecular oxygen. The first segment consists of readily soluble components such as the pyridinoprotein enzymes and the

auxiliary enzymes of the citric acid cycle, while the second is the structured, particulate portion of the chain which may be looked upon as the primary electron transport chain. The available evidence suggests that the forces which bind the first segment to the structured, second segment are relatively weak. When the first segment is detached, as by sonication of the mitochondrion, the resulting chain is similar to that of ETP or ETP_H (cf. Fig. 8).

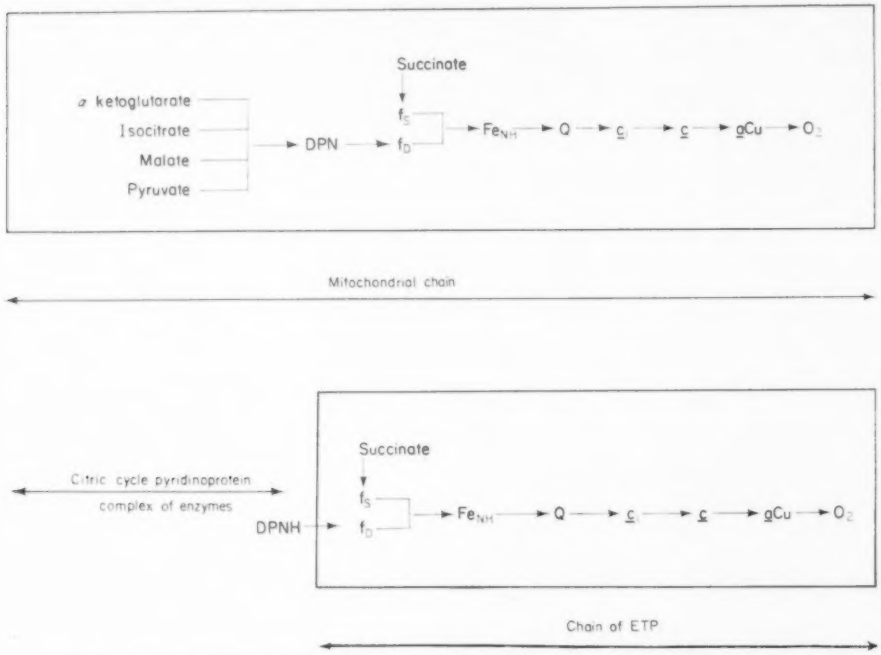


FIG. 8. The electron transport chains of mitochondria and ETP respectively—diagrammatic representations.

10. COMPONENTS OF THE STRUCTURED ELECTRON TRANSPORT CHAIN

Oxidation-reduction components

There are only five oxidation–reduction groups known to participate in the electron transport chain of ETP; namely flavin, heme, non-heme iron (abbreviated as Fe_{NH}), copper and coenzyme Q. Some of the proteins associated with these functional groups may also contain reversible oxidizable and reducible thiol groups but as yet nothing definitive can be said on that score. There are two different flavoproteins, four cytochromes, at least two forms of Fe_{NH}, one cuproprotein and only one known species of coenzyme Q. At present it is very doubtful that vitamin K plays any role in the structured electron transport chain of mitochondria from animal sources.

Flavoproteins. The succinic dehydrogenase (f_S) and the DPNH dehydrogenase (f_D) are the two flavoproteins of the structured electron transport chain. Both of these have been isolated in homogeneous state in each of several laboratories (Singer *et al.*, 1957; Basford *et al.*, 1957; Beinard, 1957; Mahler *et al.*, 1952; Ziegler *et al.*, 1959; Ringler *et al.*, 1960) and their properties which are now well documented are summarized in Table 5. The prosthetic flavin group of f_S is

TABLE 5—PROPERTIES OF ISOLATED f_S (SUCCINIC DEHYDROGENASE) AND f_D DPNH DEHYDROGENASE

	f_S	f_D
Molecular weight based on flavin content	200,000 (Singer *et al.*, 1957; Basford *et al.*, 1957)	74,000 (Ziegler *et al.*, 1959)
Prosthetic group	a dinucleotide (Kearney, 1960) not identical with FAD	FAD (Ziegler *et al.*, 1959)
Release of flavin by acid	0	+
Release of flavin by trypsin	+	0
Atoms of iron per mole of flavin	2–4 (Singer *et al.*, 1957; Basford *et al.*, 1957)	1–2 (Ziegler *et al.*, 1959)

attached to the apoenzyme by a peptide bond (Kearney, 1960). This strong linkage has frustrated efforts to detach the flavin group reversibly and to establish its exact composition. According to Kearney (1960) the functional flavin of f_S is a dinucleotide, similar to if not identical with FAD (Ziegler *et al.*, 1959) to which an amino acid side-chain is attached. The prosthetic flavin of f_D is FAD and is readily separated from the protein by treatment with acid or by heat denaturation.

Cytochromes. Keilin (1933) discovered and characterized three of the four cytochromes, viz., *a*, *b* and *c*, while Okunuki *et al.* (1941) discovered cytochrome-c_1, the fourth in the series. The functional group of each of the four cytochromes is an iron-porphyrin which is either Fe-protoporphyrin IX (as in *b*) (Person *et al.*, 1953) or a simple modification thereof (as in *c*, c_1 and *a*) (Theorell, 1938; Sekuzu *et al.*, 1960; Marks *et al.*, 1959). Cytochrome-*a* as isolated is a cuproprotein as well as a cytochrome (Griffiths *et al.*, 1961; Okunuki *et al.*, 1958; Wainio *et al.*, 1960). When cytochrome-*c* was first shown by Theorell to be a protein of M.W. 13,000 (Theorell *et al.*, 1941) it was believed that the other cytochromes were large molecular weight, insoluble proteins. This has turned out to be the case. Cytochromes-*b* and c_1 have been isolated in our laboratory in soluble form and with minimal molecular weights of 30,000 and 40,000 respectively (cf. Table 6) (Bonstein *et al.*, 1960). For reasons which will be discussed later it is likely that the correct values for the molecular weight may be even smaller even though there is no physical evidence of inhomogeneity in the preparations of these two cytochromes. The minimal molecular weight of cytochrome-*a* in a form in which

enzymatic activity is fully retained is about 100,000 (Griffiths *et al.*, 1961). Here again the probability is that the true molecular weight is considerably lower.

TABLE 6—SOME PROPERTIES OF THE ISOLATED CYTOCHROMES

	a	b	c_1	c
Properties as isolated				
Solubility in water in absence of detergents	0	0	+	+
Molecular weight			< 200,000	13,000
State	polymer	polymer	polymer	monomer
Depolymerized cytochrome				
Depolymerizing agent	thioglycollate	cetyldimethyl ethyl ammonium bromide	thioglycollate	
Solubility in water	+	+	+	
Molecular weight	100,000	30,000	37,000	
State	monomer	monomer	monomer	
Heme group	heme-*a*	Fe-protoporphyrin IX	modified Fe-protoporphyrin IX	modified Fe-protoporphyrin IX

Cytochromes-*a*, *b* and c_1 as isolated occur in the form of aggregates or polymers. Criddle & Bock (Ambe *et al.*, 1959) have shown that cytochromes-*a* and c_1 can be depolymerized to the monomeric state by anionic detergents. The monomers readily repolymerize to the original polymer or aggregate as soon as the detergent concentration is reduced below a critical level. Cytochrome-*b* as isolated is water insoluble but it forms a water-soluble monomeric compound in the presence of the cationic detergent, cetyldimethylethyl ammonium bromide (Ambe *et al.*, 1959).

Fe_{NH}. The electron transport particle (ETP) contains relatively large amounts of iron—far in excess of the iron associated with porphyrin (Crane *et al.*, 1956). Part of the iron is tightly bound to f_S (Singer *et al.*, 1957) and part to f_D (Bernard, 1957; Mahler *et al.*, 1952; Ziegler *et al.*, 1959; Ringler *et al.*, 1960), but the locale of the rest of it in the electron transport chain is still undefined. Ziegler & Doeg (1961) in our laboratory have demonstrated that a significant proportion of Fe_{NH} is rapidly reduced by either succinate or DPNH, that this reduction is inhibited by thenoyltrifluoroacetone in the case of succinate and amytal in the case of DPNH, and that the oxidation of reduced Fe_{NH} is inhibited by antimycin A. The reducibility of Fe_{NH} is one of the best diagnostics of an active particle. When this property is lost the electron transport activities of the particle are also lost.

The spectrum of Fe_{NH} in a soluble species of succinic dehydrogenase has been inferred at least qualitatively from the difference in spectrum between the enzyme in presence of substrate and the enzyme in presence of both substrate and

thenoyltrifluoroacetone which suppresses the reduction of Fe_{NH} but not of f_s (Ziegler *et al.*, in preparation). Such measurements indicate that Fe_{NH} has a broad absorption band between 425 and 475 mμ which overlaps with that of flavin. It is very likely that measurements at 450 mμ which purportedly measure only flavin turnover in fact also reflect turnover of Fe_{NH}. Beinert & Sands (Sands *et al.*, 1960; Beinert *et al.*, 1960) have characterized an Fe_{NH} complex in mitochondria and submitochondrial fragments by a specific electron spin resonance signal (g = 2). A spectrum of this signal is shown in Fig. 9. There is a different g2 iron

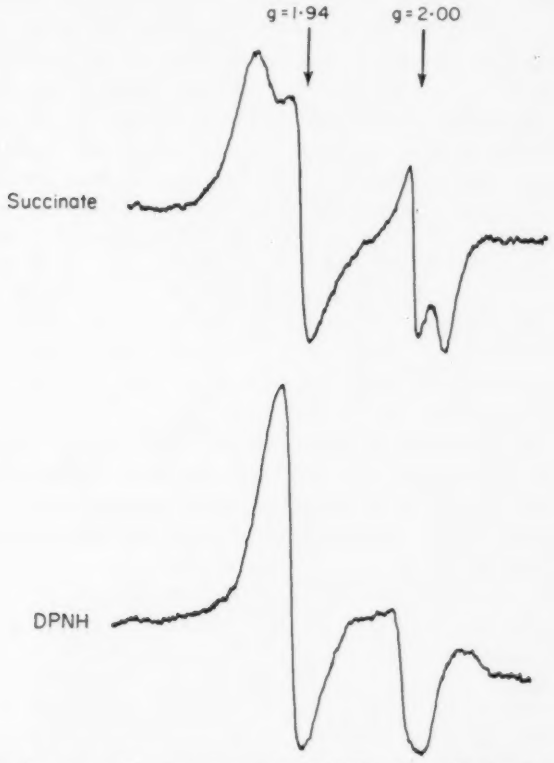


FIG. 9. The electron spin resonance signal (g2) of non-heme iron associated respectively with the succinic chain (recording in the upper half) and the DPNH chain (recording in the lower half). (Courtesy of H. Beinert.)

complex for the succinic and DPNH chains respectively—reducible only by the appropriate substrate. Reagents which interfere either with the reduction or oxidation of Fe_{NH} as determined by the direct chemical method interfere to a corresponding degree with the appearance or disappearance of the g2 signal. Although only a part of the total Fe_{NH} content of a given particle can be accounted

for in terms of the g2 signal it would appear that the reducible iron is in large measure accounted for by the complex responsible for the g2 signal.

The chemical characterization of Fe_{NH} has yet to be accomplished. The available evidence suggests that Fe_{NH} is attached to a protein and that it does not exist in the form of an organic complex. The reduced form of Fe_{NH} is extractable from its protein in glacial acetic acid (Doeg *et al.*, in preparation). Apparently it is extracted in the form of free iron. p-Chloromercuribenzoate displaces Fe_{NH} from the protein with which it is associated and this displacement reaction would suggest the attachment of Fe_{NH} to some protein-bound sulfhydryl group.

Copper. There is an invariant association between cytochrome-*a* and copper (Griffiths *et al.*, in press; Takemori, 1960). For each mole of heme-*a* there is one atom of copper. Both the heme and the copper are associated with the same protein which may be characterized as a cuprohemoprotein. It is possible to extract the heme from the protein with acid acetone without extracting the copper. Thus there are independent modes of attachment to the proteins for the two functional groups. Some indirect evidence suggests that in addition to their binding to the protein the two oxidation groups are chelated to one another—the copper to the side chain of the iron porphyrin (Griffiths *et al.*, 1961).

The cytochrome-*a*–copper complex catalyzes the oxidation of reduced cytochromes by molecular oxygen and may be identified as the terminal portion of the electron transport chain. It has long been supposed that there are two cytochromes responsible for the cytochrome oxidase reaction (Keilin *et al.*, 1939). But the weight of evidence now favors the idea of a single cytochrome entity, viz., the cytochrome-*a*–copper complex.

Chemical and physical methods have been used to demonstrate that copper undergoes rapid oxidoreduction and that this turnover rate is compatible with a central role for Cu in the oxidation of reduced cytochrome-*c* by oxygen. It is probable that copper is the catalytic link between the heme group and molecular oxygen (Griffiths *et al.*, 1961).

Coenzyme-Q. The substituted benzoquinone known as coenzyme-Q was discovered independently by Morton and his colleagues at Liverpool (Morton *et al.*, 1957, 1961) and by Crane, Lester, Hatefi & Widmer (Crane *et al.*, 1957; Wolf *et al.*, 1958) of the Enzyme Institute. The structure (shown in Formula 1) was established by Folkers and his group at the Merck, Sharpe and Dohme Laboratories (Wolf *et al.*, 1958; Folkers, 1961) and by Isler of the Hoffman–La Roche Laboratories in Basel (Morton *et al.*, 1958; Isler *et al.*, 1961). The length of the side chain varies from 6–10 isoprenoid units depending upon the source of the coenzyme (cf. Table 7) (Lester *et al.*, 1959).

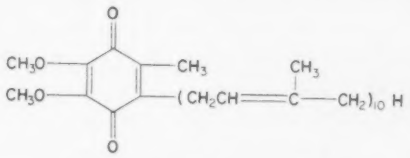
Coenzyme-Q is a component in the electron transport chain between both succinate and DPNH on the substrate side and molecular oxygen on the terminal sector of the chain (Green *et al.*, 1959; Redfearn *et al.*, 1959, 1961). When coenzyme-Q is extracted from respiratory particles, activity is lost and this activity can be restored by adding back the coenzyme (Lester *et al.*, 1959). The rate of reduction and oxidation of the coenzyme is of the correct order of magnitude for

a central role in electron transport. Furthermore, reagents which inhibit overall electron transport affect correspondingly either the reduction or oxidation of coenzyme-Q (Hatefi, 1959).

TABLE 7—HOMOLOGUES OF COENZYME Q (UBIQUINONE)

Source	Number of isoprenoid units	Designation
Beef heart	10	Coenzyme Q_{10} (Ubiquinone$_{50}$)
Torula utilis	9	Coenzyme Q_9 (Ubiquinone$_{45}$)
Azotobacter vinelandii	8	Coenzyme Q_8 (Ubiquinone$_{40}$)
Torula utilis	7	Coenzyme Q_7 (Ubiquinone$_{35}$)
Saccharomyces cerevisiae	6	Coenzyme Q_6 (Ubiquinone$_{30}$)

Data of Lester, Hatefi, Widmer & Crane (1959).
See also Isler, Rüegg, Langemann, Schudell & Ryser (1961), Morton *et al.* (1961)



Formula 1. Coenzyme Q_{10}.

In mitochondria from whatever source, coenzyme-Q is invariably present (Lester *et al.*, 1959). It is only among the particles of bacteria that there are exceptions to this rule. The particles derived from *Mycobacterium phlei* do not contain coenzyme-Q but contain a derivative of vitamin K which, according to Brodie (Brodie *et al.*, 1960), fulfils a central role in electron transport and oxidative phosphorylation in this organism. The relation between coenzyme-Q and vitamin K in bacteria which contain both has yet to be clarified. At present there appears to be no direct evidence that vitamin K is a functional component of the electron transport chain of animal mitochondria.

Lipids of the electron transport chain

The mitochondrion contains some 28 per cent weight of lipid and this ratio of lipid to protein remains constant in all the structured particles into which the mitochondrion can be fragmented, according to Fleischer & Klouwen (Fleischer *et al.*, in preparation). The composition of mitochondrial lipid is summarized in Table 8. It has been shown by Fleischer *et al.* (in preparation) that the lipid

composition is essentially identical for various types of submitochondrial particles. Such small differences that have been observed could be referable to redistribution of lipid during fractionation. As discussed briefly in the earlier part of this lecture, the twin unit of the electron transport chain may be conceived of as a rod-like

TABLE 8—COMPOSITION OF LIPID IN MITOCHONDRIA AND DERIVATIVE PARTICLES

	% of total P				
	Phosphatidyl inositol	Phosphatidyl choline	Phosphatidyl ethanolamine	Cardiolipin	Residue
Mitochondria	8	41	33	15	1
ETP_H	10	42	34	15	1
Cytochrome oxidase	10	25	25	29	10
Succinic-*c* reductase	14	37	26	15	8
DPNH-*c* reductase	12	31	30	23	5
Succinic-Q reductase	16	51	23	7	2

Data of Fleischer, Brierly & Klouwen (Fleischer *et al.*, 1961).

ordered pair of structures with a lipid interior and this lipid is the medium in which the oxidoreductions of the structured segment of the electron transport chain take place.

In addition to serving as the reaction medium the lipid also contains two mobile oxidation–reduction substances, viz., coenzyme-Q and cytochrome-*c*. These substances, which are readily extracted from and reinserted into the lipid of particles, appear to be capable of shuttling electrons between pairs of fixed oxidation–reduction components. Cytochrome-*c* as isolated is a water-soluble protein but it is capable of reacting with lipid to form a lipid-soluble, water-insoluble complex (Ambe *et al.*, 1959; Michelazzi, 1955). The so-called bound cytochrome-*c* is probably a lipid complex of cytochrome-*c*.

The phospholipides of mitochondria can readily assume an ordered state in which they form micelles of molecular dimensions, and these micelles are so finely dispersed in water that for all practical purposes the lipid may be considered to be miscible with water. This property may be of considerable physiological significance since it provides a mechanism for the rapid exchange of solutes between the water and lipid phases of the mitochondrion.

The structural protein

More than 50 per cent of the total protein of the electron transport particle can be accounted for by a water-insoluble, colorless protein which contains no oxidation–reduction groups and which can be isolated in a lipid-free form (Green *et al.*, 1961). This protein will be referred to as the structural protein. The isolated structural protein in its aggregated or polymeric form can be depolymerized in alkali or in 67 per cent acetic acid to a water-soluble species of molecular weight

between 20,000 and 30,000 (Criddle *et al.*, in press). There should be as many as 50 of such monomers in the unit of the electron transport chain. Cytochromes-*a*, *b* and c_1 occur in the form of tight, large molecular weight complexes with the structural protein and this in part accounts for the insolubility of these cytochromes in the crude state. The separation of these cytochromes from one another is relatively simple but the main technical problem is that of separating each of these cytochromes from the structural protein.

Structural protein also forms tight complexes with lipid but the separation of structural protein from lipid is much easier to accomplish than its separation from the hemoproteins.

The present working hypothesis is that the structural protein forms the coat of the structural unit within which both lipid and cytochromes are imbedded, and to which lipid and the three cytochromes (*a*, *b* and c_1) are attached. The particulate, structured part of the electron transport chain is thus the locale of the structural protein. Studies on the structural protein have established that the mitochondrion and submitochondrial particles are held together largely through hydrophobic bonds. Extensive non-polar regions in the structural protein as well as in the three cytochromes probably underlie the high affinity of these cytochromes for the structural protein and the great tendency of the monomeric species of each of these proteins to polymerize to form polymers in aqueous solution. Furthermore, these proteins with extensive non-polar regions have a high affinity for lipid. Thus the network of structural protein, lipid and bound cytochromes is an expression of the powerful adhesive tendencies of hydrophobic bonds. The strength of these bonds may approach in magnitude that of a covalent bond (5000–6000 cal.).

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TABLE 9—COMPOSITION OF THE ELECTRON TRANSPORT PARTICLE (ETP) OF BEEF HEART MITOCHONDRIA

Component	mμM/mg protein or mμatoms/mg protein	Molecular or atomic ratio
Flavin of f_S	0·32	1
Flavin of f_D*	0·32	1
Cytochrome-*a*	1·30	4
Cytochrome-*b*	0·68	2
Cytochromes ($c+c_1$)†	0·60	2
Fe_{NH}	8·5	27
Copper	2·5	8
Coenzyme-Q	4·9	15

* f_D is equated with acid extractable flavin.

† Since there is a considerable amount of cytochrome-*c* in ETP the amount of c_1 would be insufficient for a 1 : 1 ratio of c_1 to *b*. Data from less complex particles suggest that the above estimate of the c_1 concentration in ETP is too low.

Data of Crane, Glenn & Green (1956), Linnane & Ziegler (1958), Griffiths & Wharton (in press), Doeg & Ziegler (in preparation).

11. COMPOSITION OF ETP

The molecular proportions of the various oxidation–reduction components in ETP (Green, 1959) are summarized in Table 9. It is to be noted that not all of Fe_{NH} or copper can be implicated in electron transport. Probably no more than 50–60 per cent of the total iron or 66 per cent of the copper is functional. The rest may either be adventitious metal present in the preparation or else metal which fulfills some role other than that of oxidation–reduction. Coenzyme-Q and Fe_{NH} account for the highest proportion of oxidation–reduction equivalents. The two flavoproteins and three of the cytochromes (*b*, c_1 and *c*) are in 1 : 1 molecular proportions to one another. But there is twice as much cytochrome-*a* as each of the other cytochromes. It should be emphasized that in ETP we are dealing with constant molecular proportions but not necessarily with stoichiometry, for the latter would apply only when two members of the chain are directly attached one to the other.

12. SEQUENCE AND ARRANGEMENT OF THE ELECTRON TRANSPORT CHAIN

There is a wealth of experimental evidence to suggest that the structured electron transport chain is made up of two intercommunicating but separable chains—one for oxidation of succinate and the other for oxidation of DPNH (Hatefi *et al.*, 1961; Green *et al.*, 1961; Mackler *et al.*, 1954; Crane *et al.*, 1957). Each chain consists of the appropriate flavoprotein, Fe_{NH}, coenzyme-Q, and cytochromes-c_1, *c* and *a* as shown below (arrows indicating the direction of electron flow and the dotted lines symbolizing the intercommunication of the chains):

$$\begin{array}{l} f_S \rightarrow Fe_{NH} \rightarrow Q \rightarrow bc_1 \rightarrow c \rightarrow (a\text{-}Cu)_2 \\ \quad\vdots\quad\ \vdots\quad\ \vdots\ \vdots \\ f_D \rightarrow Fe_{NH} \rightarrow Q \rightarrow bc_1 \rightarrow c \rightarrow (a\text{-}Cu)_2 \end{array}$$

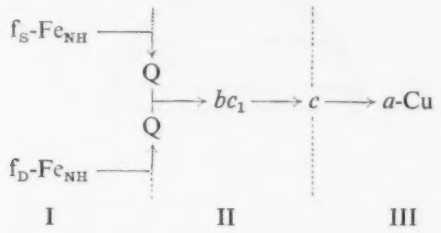
There are one or more functional as well as structural links between these two chains which permit electrons to flow from one chain to the other. We may think of each of the two electron transport chains (succinic and DPNH) as made up of three fixed sections:

	I	II		III
Succinic	f_S-Fe_{NH}	Q	bc_1	*c*	*a*Cu
DPNH	f_D-Fe_{NH}	Q	bc_1	*c*	*a*Cu

(1) the flavoprotein–Fe_{NH} section; (2) the bc_1 section; and (3) the *a*-Cu section. Coenzyme-Q is the mobile link between I and II, and cytochrome-*c* the mobile link between II and III. Sections II and III make up the structured and particulate segment of the electron transport chain. Cytochromes-c_1 and *a* are contained within the lipid phase while the flavoprotein, though attached to lipid, is external

to the capsule. Cytochromes-*a*, *b* and c_1 are bonded to the structural protein whereas the flavoprotein is not.

It has yet to be rigorously excluded that there is only one chain with the f_S–Fe_{NH} and f_D–Fe_{NH} sections merging with section II as shown in the diagram below:



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The bulk of available evidence does not support this interpretation but on the other hand the proof for two separate chains is by no means conclusive.

In the crista and the external membrane of the mitochondrion the units of the electron transport chain come in lateral pairs to form a sandwich as it were (cf. Fig. 10). It is this sandwich arrangement which accounts for the triple-layer effect of the membrane systems. Thus one has to distinguish between the capsular or sandwich arrangement of twin chains as in the mitochondrion or ETP_H and the unpaired arrangement of the chains as in ETP. The twin chain or capsular arrangement is a *sine qua non* for oxidative phosphorylation whereas electron transport does not necessarily depend upon this particular arrangement.

The lipid layer provides a device for intercommunication not only between paired units but also between stacked units. Coenzyme-Q and cytochrome-*c* can move in all directions through the lipid and these are probably the molecular instruments by which electrons entering a given chain can radiate to the components not only of this chain but of all other chains which abut on the same lipid layer. This intercommunication of chains is the most characteristic feature of the electron transport chain, and one of the merits of the hypothesis formulated above is that it provides an adequate explanation for this phenomenon.

The separation of the section containing cytochrome-*a* from that containing cytochrome-c_1 is easily accomplished by deoxycholate, cholate or various alcohols (Hatefi *et al.*, 1961; Green *et al.*, 1961; Crane *et al.*, 1957). The ease with which these two sections can be separated would suggest an arrangement in which a lipid layer separates and binds the two sections. The separation of the flavoprotein from the middle section is also readily achieved (Basford *et al.*, 1957; Bernard, 1957; Mahler *et al.*, 1952). Reagents such as cholate-salt or short-chain alcohols and exposure of particles to acetone or heat are effective for this purpose. The separation of cytochrome-*b* from c_1 is by far the most difficult to achieve without sacrifice of enzymatic activity. With fine control of conditions it has been found possible to remove cytochrome-*b* from the succinic chain and still retain a significant amount of electron transport activity (Green *et al.*, 1959).

Since lipid is in large measure the cement which holds the chain together, it is of interest to consider the fate of lipid when components are detached from the chain. The succinic or DPNH flavoproteins can be detached from the chain in a

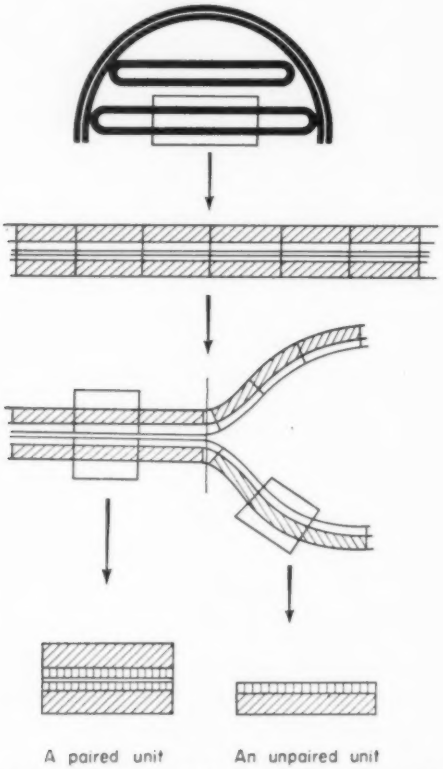

FIG. 10. Paired and unpaired units of the electron transport chain. The units in crista (upper diagram) are paired as shown diagrammatically in the middle diagram. The lower diagram shows the transition from paired chains (left) to unpaired chains (right).

form which does not contain lipid. But it is also possible to effect the detachment in a way which brings the flavoprotein out still associated with a considerable amount of lipid (Ziegler *et al.*, 1959; Ziegler *et al.*, in preparation). There is probably a layer of lipid which separates the succinic from the DPNH chains since lipid is released whenever the two chains are separated (Green *et al.*, 1961; Fowler *et al.*, in preparation). When the cytochrome-*a* segment is separated from the cytochrome-bc_1 segment the lipid at the interface between the two segments usually remains with the bc_1 section (Green *et al.*, 1954).

13. FRAGMENTATION OF THE MITOCHONDRION AND OF THE ELECTRON TRANSPORT CHAIN

The structure of the mitochondrion already suggests in principle the way in which it can be broken down stepwise into progressively less complex units. We may categorize the various types of fragmentation under four headings (cf. Table 10): (1) disruption of the mitochondrion into fragments containing intact units

TABLE 10—MODES OF FRAGMENTATION OF THE MITOCHONDRION

1. Mechanical disruption by grinding or sonication

mitochondria ⟶ ETP_H (twin chains) ⟶ ETP (unpaired chains)

2. Separation of succinic and DPNH chains

ETP → succinic oxidase
ETP → DPNH oxidase

3. Fragmentation of whole chain into a cytochrome-*c* reductase and a reduced cytochrome-*c* oxidase

succinic oxidase → succinic-*c* reductase
succinic oxidase → cytochrome oxidase
DPNH oxidase → cytochrome oxidase
DPNH oxidase → DPNH-*c* reductase

4. Fragmentation of succinic and DPNH-*c* reductase

succinic-*c* reductase → succinic-Q reductase
succinic-*c* reductase → QH_2-*c* reductase
DPNH-*c* reductase → QH_2-*c* reductase
DPNH-*c* reductase → DPNH-Q reductase

either with twin chains as in ETP_H or unpaired chains as in ETP; (2) separation of the succinic from the DPNH chains; (3) resolution of each chain into a red heme fragment containing f, *b*, c_1 and *c* and a green heme fraction containing *a*Cu; and (4) resolution of the red heme fraction into a flavin-Fe_{NH} and a bc_1 subfraction. These are the generalized procedures and most methods involve combinations of all four categories.

The stepwise simplification may be visualized in yet another way as depicted in Fig. 11. Each oxidation–reduction component on the main chain may be considered to be a potential dividing point leading to two particles—one of which catalyzes the reduction of this component and the other the oxidation of this

component. Thus, there is a cytochrome-*c* reductase and a reduced cytochrome-*c* oxidase; a coenzyme-Q reductase and a reduced coenzyme-Q oxidase. But this strategy cannot always be employed since only coenzyme-Q and cytochrome-*c* can be tested by adding them back to particles. With cytochromes-*b*, c_1 or *a* this

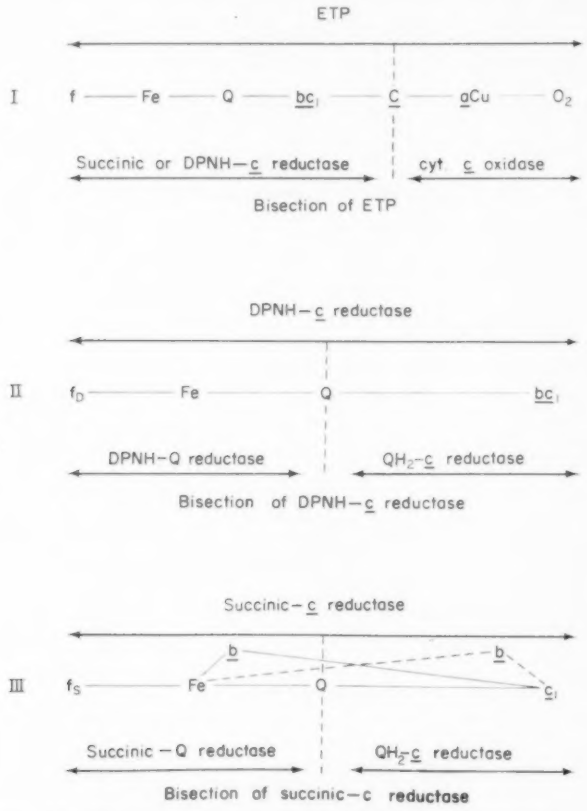

FIG. 11. Stepwise bisection of the electron transport chain.

procedure is ruled out. By the method available the fixed protein components cannot be removed from particles and then reinserted. Thus one must distinguish between the theoretically possible cleavage points and the *de facto* cleavage points. The point of cleavage for functional particles is always at the point in the chain where the mobile components (Q and *c*) intervene.

The fragmentation of particles can be extended to the point where all components are torn apart one from the other and then the individual entities isolated. This has now been done for each of the components of the electron transport system.

14. REAGENTS FOR FRAGMENTATION

Relatively few useful reagents have been uncovered for the effective fragmentation of the electron transport chain (cf. Table 11) and without exception these are

TABLE 11—REAGENTS FOR FRAGMENTATION OF MITOCHONDRIA AND SUB-MITOCHONDRIAL PARTICLES

Bile acids—cholate, deoxycholate (in presence of salt)
Alcohols—t-amyl, isobutyl, butyl
Detergents—dodecylsulfate, cetyldimethylethyl ammonium bromide
Hydrocarbons—cyclohexane, isooctane, petroleum ether

all variations on a single theme, the theme being that lipid is involved in the bonds which hold the chain together. The detergents, bile acids (Neufeld *et al.*, 1954; Wainio *et al.*, 1956), lower chain alcohols (Green *et al.*, 1954) and hydrocarbons (Ziegler *et al.*, in preparation; Fowler *et al.*, in preparation; Ziegler *et al.*, 1959) are the only known classes of compounds which can be used effectively for fragmentation of the chain without sacrifice of enzymatic activity in the daughter particles. The hydrophobic and electrostatic bonds which bind lipid to protein and also protein to protein are undoubtedly the targets of the effective fragmenting agents. We have much to learn about the nature and stability of these bonds before the art of fragmenting the mitochondrion can graduate to a science.

In this context it is of interest to point out that some of the most potent inhibitors of electron transport activity are relatively water-insoluble lipid-soluble molecules, e.g. antimycin A (Reif *et al.*, 1953), thenoyltrifluoroacetone (Ziegler *et al.*, 1961), 2-hexyl 4-hydroxy quinoline-N-oxide (Lightbown *et al.*, 1956) and the antimalarial napthoquinone (SN5949) (Ball *et al.*, 1947).

15. SITES OF ACTION OF KEY INHIBITORS OF THE ELECTRON TRANSPORT SYSTEM

Over the years, reagents have been discovered which specifically inhibit electron flow through a given site in the electron transport chain (Fig. 12). Cyanide, azide and H_2S are specific inhibitors of the oxidation of reduced cytochrome-*c* by molecular oxygen (Keilin, 1933) and it is likely that these combine with copper or heme-*a*. Antimycin A and 2-hexyl 4-hydroxy quinoline-N-oxide are specific inhibitors of the reduction of cytochrome-c_1—a reduction which may be metal catalyzed (Chance *et al.*, 1956; Lightbown *et al.*, 1956). Amytal (Ernster *et al.*, 1955) inhibits the reduction of Fe_{NH} only of the DPNH chain (Hatefi *et al.*, in press) while thenoyltrifluoroacetone is specific for Fe_{NH} of the succinic chain.

16. CITRIC CYCLE AND AUXILIARY ENZYMES

The mitochondrion may be looked upon as a composite of three elements: (1) the structured, repeating electron transport chains; (2) auxiliary enzymes which are attached to but external to the structured chains; (3) enzymes which have neither a functional nor a structural relation to the electron transport chains. The enzymes of the third group may in large measure perform a maintenance service.

These can catalyze the synthesis of components essential for mitochondrial activity such as phospholipides and cytochrome-*c* and such syntheses can proceed in the spaces between cristae. The enzymes of the second group are present in constant

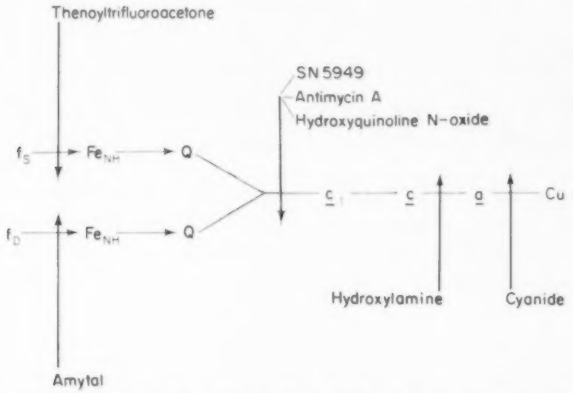

FIG. 12. Site of action of the key inhibitors of the electron transport chain. The following sites of action have been established: amytal at the Fe_{NH} of the DPNH chain; thenoyltrifluoroacetone at the Fe_{NH} of the succinic chain; SN5949, antimycin A and nonyl hydroxyquinoline-N-oxide at some component between Q and c_1; hydroxylamine at the heme of cytochrome-*a*; and cyanide at the copper of cytochrome-*a*.

molecular proportions and are arranged in a non-random fashion. The constancy of the citric cycle oxidative of mitochondria bespeaks a precise molecular relation of citric cycle enzymes to components of the structured electron transport system. There is evidence from many lines of investigation that all these auxiliary enzymes are arranged in a precise pattern just as are the components of the electron transport chain and that each electron transport unit has its fixed complement of auxiliary enzymes.

The internal arrangements of several of the auxiliary enzymes have been examined in detail by various investigators. The two complexes of enzymes which catalyze respectively the oxidative decarboxylation of α-ketoglutarate and pyruvate by DPN have been shown to be composites of at least four different enzymes and four cofactors (Searls *et al.*, 1959; Gunsalus *et al.*, 1957; Reed, 1957; Massey, 1960; Schweet *et al.*, 1951). The affinity of the component enzymes in these complexes for one another is of such a high order of magnitude that only rather drastic measures suffice to resolve them into their component enzymes (Massey, 1960). The α-ketoglutaric dehydrogenase complex is of particular interest in that it illustrates several important principles: (1) the principle of a soluble organized complex of enzymes; (2) the principle of a series of oxidation–reduction reactions taking place within a soluble complex in which the freedom of movement of the individual components is greatly restricted; (3) the principle of the

arrangement of enzymes around one or more coenzymes to make possible sequential enzymatic reactions.

The two final steps in the oxidation of α-ketoglutarate by DPN is the reduction of a flavoprotein by bound reduced lipoic acid and the oxidation of the reduced flavoprotein by DPN. The flavoprotein with lipoic dehydrogenase activity has been shown by Massey (Massey, 1960) and Sanadi (Schweet *et al.*, 1951) to contain a dithiol group and they have demonstrated that the sequence of electron flow is as follows:

$$\text{reduced lipoic} \rightarrow \text{dithiol group of flavoprotein} \rightarrow \text{flavin} \rightarrow \text{DPN}$$

The overall sequence for electron flow within the α-ketoglutaric dehydrogenase complex would then involve four oxidoreduction reactions between the following five components:

$$\text{succinsemialdehydo-diphosphothiamine} \rightarrow \text{lipoic} \rightarrow \text{enzyme dithiol} \rightarrow \text{flavin} \rightarrow \text{DPN}$$

The enzymes which implement the fatty acid β-oxidation cycle are also arranged in some form of complex but since this complex is more readily dissociated than the α-ketoglutaric dehydrogenase complex it has not yet been systematically studied (Green, 1957).

The organizational devices by which the citric cycle and auxiliary enzymes are arranged with respect to the structured electron transport chain is a relatively unexplored area which should richly repay closer examination. One facet of particular relevance to oxidative phosphorylation is the state of the so-called bound mitochondrial coenzymes such as DPN, ATP, lipoic acid, diphosphothiamine and CoASH (Green, 1951). A group of enzymes share each of these cofactors and there are many indications that these cofactors exist in some bound form. Recently in our laboratory the mitochondrial β-hydroxybutyric dehydrogenase was finally solubilized and it was found that the interaction of this enzyme with DPN required lecithin as cofactor (Jurtshuk *et al.*, 1961). The function of the cofactor could be that of complexing the enzyme with DPN.

17. RECONSTRUCTION OF ELECTRON TRANSPORT ACTIVITY

In view of the complexity of the structure of the electron transport system, one might wonder to what extent the whole can be reconstructed from the parts. Reconstruction in the sense that an enzyme system can be functionally coupled to another is very easily achieved with soluble as well as with particulate enzymes. The only requirement in such cases is that the two systems should share a water-soluble intermediate, which must be present during assay at relatively high concentration. For example, when succinic–cytochrome-*c* reductase is supplemented with cytochrome oxidase and cytochrome-*c*, succinic oxidase activity can be demonstrated. In this case the intermediate is the water-soluble component, cytochrome-*c* which is required at relatively high concentrations in order to shuttle back and forth between the reductase and the oxidase to achieve a functional link.

What we are concerned with in the present context is an actual recombination of particles to form a single functional entity. Hatefi and his colleagues (Hatefi *et al.*, 1961) in our institute have achieved this for the first time. They have reconstructed DPNH–cytochrome-*c* reductase, succinic–cytochrome-*c* reductase as well as DPNH and succinic–cytochrome-*c* reductase by mixing together the following enzyme preparations (cf. Table 12, section A):

(I) DPNH—coenzyme-Q reductase,
(II) succinic—coenzyme-Q reductase,
and (III) reduced coenzyme-Q—cytochrome-*c* reductase.

TABLE 12—RECONSTRUCTION OF DPNH AND SUCCINIC–CYTOCHROME-*c* REDUCTASE SYSTEMS

		Specific activity	
	Enzymes	Succinic–cyt.-*c*	DPNH–cyt.-*c*
A	DPNH·Q reductase (I)	0·0	6·0
	Succinate-Q reductase (II)	0·0	0·0
	QH^2-*c* reductase (III)	0·2	0·0
B	I + III	0·3	30·5
	II + III	27·0	0·0
	II + III + Q	45·0	0·0
C	I + II + III	43·2	17·0
	I + II + III + Antimycin-A	0·0	0·0
	I + II + III + Amytal	40·0	0·0
	I + II + III + Thenoyltrifluoroacetone	0·0	11·0

In all cases enzymic activity was measured at 1 μg protein/ml. Specific activity is expressed as μmole cytochrome-*c* reduced per min per mg protein of I or II at 38°.

Each enzyme preparation is essentially free of activities other than that mentioned above. When (I) and (III) are combined (cf. Table 12, section B), a highly active DPNH–cytochrome-*c* reductase system is reconstructed, which responds fully to the specific inhibitors, amytal and antimycin-A. Combination of (II) and (III) results in a succinic–cytochrome-*c* reductase whose activity is inhibited by thenoyltrifluoroacetone and antimycin-A. And when all three fractions are mixed together, both the DPNH–cytochrome-*c* and the succinic–cytochrome-*c* reductase systems are reconstructed with characteristic response to the above inhibitors of the electron transport system (cf. Table 12, section C). The reconstructed DPNH–cytochrome-*c* reductase system contains a sufficient amount of bound coenzyme-Q, while the reconstituted succinic–cytochrome-*c* reductase is deficient in this respect. This deficiency is due to the low concentration of coenzyme Q in the succinic–coenzyme-Q reductase enzyme, and is rectified by supplying coenzymes-Q to the reconstituted succinic–cytochrome-*c* reductase system (cf. Table 12, section B).

It is rather remarkable that when the three particles are mixed together, then the succinic-Q reductase moiety can make use of coenzyme-Q contained in the DPNH-Q reductase segment and now operate at full capacity (cf. sections B and C of Table 12). These dramatic examples point to the possibility that eventually the entire electron transport chain can be reconstructed from the component units.

The conditions for recombination of the daughter particles to form the original parent particle in the reconstruction experiments of Hatefi *et al.* are of particular interest. The recombination takes place only in relatively concentrated solutions of the two particles (cf. Table 13). Once the recombination has been consummated

TABLE 13—EFFECT OF CONCENTRATION ON RECONSTRUCTION OF SUCCINIC–CYTOCHROME-*c* AND DPNH–CYTOCHROME-*c* REDUCTASE SYSTEMS

I Succinic-Q reductase (μg/ml)	II DPNH-Q reductase (μg/ml)	III QH_2-*c* reductase (μg/ml)	Specific activity
1	—	1	0·9
10	—	10	2·6
100	—	100	13·2
6000	—	6000	39·0
—	1	1	4·5
—	10	10	6·4
—	5000	5000	43·2

Specific activity is expressed as μmoles cytochrome-*c* reduced per min per mg protein of I or II.

After mixing the enzymes at concentrations indicated, the solutions were diluted and activity measured at 1 μg protein/ml.

the emergent particle does not dissociate in dilute solution. But it will not form to any appreciable extent except in concentrated solutions of the daughter particles.

There is yet another type of reconstruction which involves separating out from a particle one component such as cytochrome-*c* (Jacobs *et al.*, 1955) or coenzyme-Q (Green *et al.*, 1959) or a flavoprotein (Beinert *et al.*, 1961) and then restoring activity by adding back the particular component extracted. This type of reconstruction is of a somewhat different category than that for the recombination of two separate particles.

The classical biochemist had long entertained the hope that all mitochondrial activities could be reconstructed by isolating each of the cytochromes, flavoproteins, etc., and then by putting them together in the proper proportions and under suitable conditions to realize the same enzymatic properties from this mixture of pure components that characterize the intact mitochondrion. This hope is unrealistic in that it takes no cognizance of the fact that the components of the electron transport chain are part of a structured milieu and that unless this structure or its equivalent is reproduced, reconstruction lies outside the realm of possibility.

18. MECHANISM OF ELECTRON TRANSPORT

The oxidation of succinate of DPNH by oxygen involves basically three concerted oxidation reduction sequences which take place seriatim in the three segments of the electron transport chain:

$$\text{I} \qquad \text{succinate} \xrightarrow{1} \text{flavin} \xrightarrow{2} \text{Fe}_{\text{NH}} \xrightarrow{3} \text{Q},$$

$$\text{II} \qquad \text{QH}_2 \xrightarrow{1} bc_1 \xrightarrow{2} c,$$

$$\text{III} \qquad \text{reduced } c \xrightarrow{1} a \xrightarrow{2} \text{Cu} \xrightarrow{3} \text{O}_2$$

Let us consider the first segment of the structured electron transport chain which contains the flavin-Fe_{NH} complex. According to the above formulation this complex combines with the reducing substrate (succinate or DPNH) and the acceptor (CoQ). Then a wave of oxidoreductions takes place within this complex leading to the transfer of two electrons from the substrate to coenzyme-Q. There are at least three separate oxidoreductions in this particular sequence. The QH_2 formed in the first concerted reaction dissociates from the first segment and then combines with the second segment which contains cytochromes *b* and c_1. Four oxidation–reduction components are involved in the sequence catalyzed by the c_1 segment: QH_2, *b*, c_1 and *c*. Again a wave of oxidoreductions takes place leading to the transfer of electrons from QH_2 to cytochrome-*c*. Reduced cytochrome-*c* formed in this second sequence dissociates from the complex and combines with the *a*-Cu complex, the third and last in the chain. In the third sequence involving four oxidation–reduction components, electrons from reduced cytochrome-*c* reduce oxygen to water in four successive waves.

The transfer of electrons within each of the three segments of the electron transfer chain as well as the transfer of electrons within the α-ketoglutaric and pyruvic dehydrogenase complexes involves an intrinsically novel chemical principle. These complexes behave as if they were one huge resonating system in which the oxidation–reduction components share electron orbitals. But such is clearly not the case for all the components of these complexes. The components are admittedly fixed in position but it would be impossible for all of them to be in close enough molecular proximity so as to share electrons. It could be postulated that there is sufficient freedom of molecular manœuvre within the complex to permit interaction by collision. But it is far more likely that the disposition of oxidation–reduction components within an organized complex admits of a mechanism which is neither that of a resonating system with hybridized orbitals nor that of a system involving only random collisions. There is probably no precedence in classical chemistry for the mechanism of electron flow through these complexes. It has been suggested by my colleagues, R. Bock and R. Criddle, that the wave of sequential oxidoreductions which moves through the *a*-ketoglutaric dehydrogenase complex involves the pendulum action of lipoic acid and flavinadenine

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dinucleotide, both of which are anchored to their respective apoprotein by an elastic link. The rotation of the coenzyme made possible by this elastic link permits of successive collisions with other functional groups in the complex. Figs. 13 and 14 are diagrammatic representations of the Bock–Criddle hypothesis.

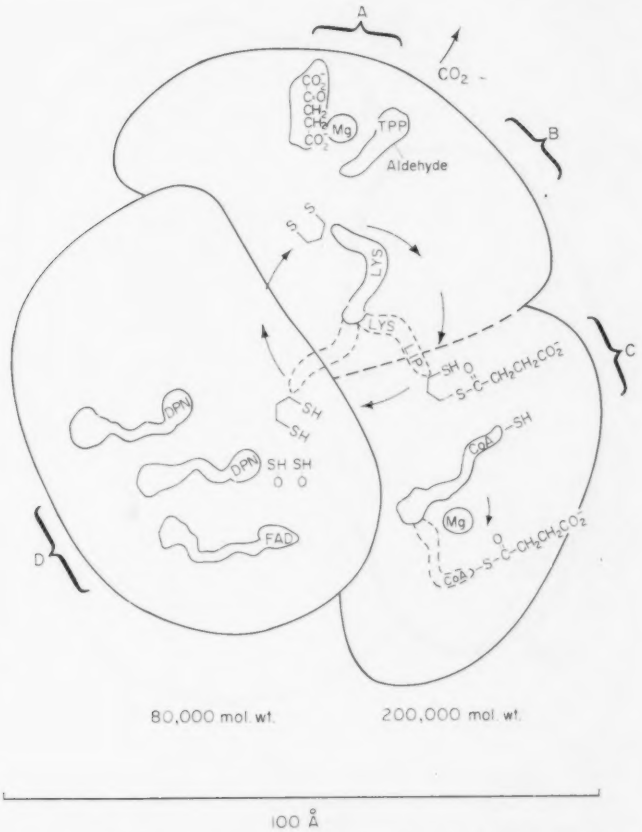

FIG. 13. A schematic representation of the central role which lysine–bound lipoic acid may play in the *a*-ketoglutaric dehydrogenase complex.

Region A: *a*-Ketoglutarate reacts with thiamine pyrophosphate (TPP) to form the succinsemialdehyde adduct of TPP with release of CO_2.

Region B: Oxidized lipoic acid interacts with the succinsemialdehyde adduct of TPP to form succinyl lipoic acid and TPP.

Region C: Succinyl lipoic acid reacts with reduced CoQ to form succinyl CoA and reduced lipoic acid.

Region D: Reduced lipoic acid reacts with an enzyme-bound disulfide group to form oxidized lipoic acid and reduced dithiol. This completes the lipoic cycle.

Region E: Electrons are transferred from reduced dithiol (of the lipoic dehydrogenase) to FAD, and from FAD to DPN. (Courtesy of R. M. Bock and R. S. Criddle.)

The EPR and spectrophotometric studies of Beinert in our institute (Keilin *et al.*, 1960) have firmly established that the interaction of either f_D or f_S with its appropriate substrate or of the flavoprotein which catalyzes the oxidation of fatty

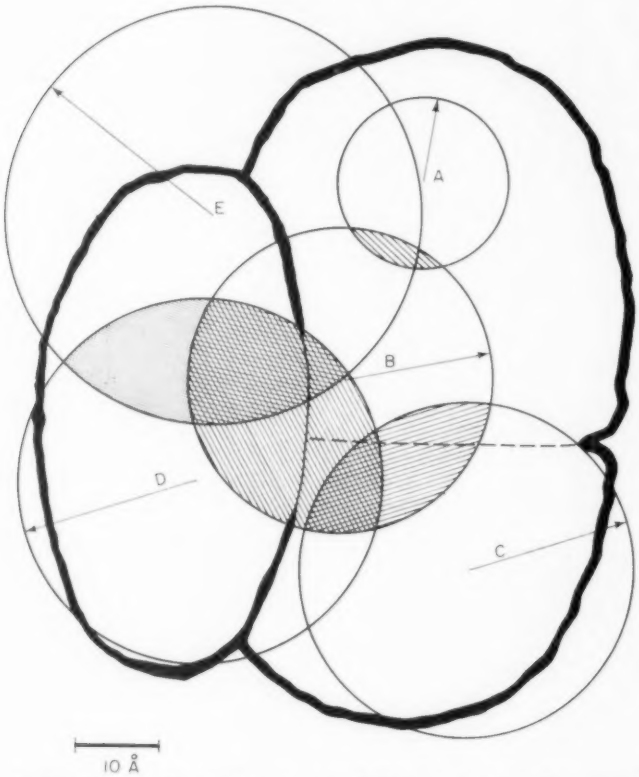

FIG. 14. Scale diagram illustrating the possibilities for physical contact between bound coenzymes attached to widely spaced points on the surface of *a*-ketoglutaric dehydrogenase complex. The following are the keys to the various symbols:

A—thiamine pyrophosphate
B—lipoic acid bound to the epsilon amino group of lysine
C—coenzyme A
D—FAD
E—DPN

Radii of the circles represent the distance between the functional point of the coenzyme and its point of attachment to the enzyme. It is assumed that A is bound to protein via the pyrophosphate group, B via the carboxyl of lysine, and C, D and E via adenine. The protein is drawn to scale assuming a molecular weight of 80,000 for the lipoic dehydrogenase and 200,000 for the rest of the *a*-ketoglutaric dehydrogenase. The volume assigned to protein includes water of hydration equal to 50 per cent of the weight of protein. (Courtesy of R. M. Bock and R. S. Criddle.)

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acyl CoA esters with its appropriate substrates leads to the formation of stable free radical or semiquinones. Thus it would appear that two-electron oxidations are carried out stepwise in the electron transport chain.

19. OXIDATIVE PHOSPHORYLATION

Although it should be obvious that the mechanism of oxidative phosphorylation is inextricably tied up with the mechanism of electron transport almost two decades of intensive effort have been directed at elucidating the former without a full knowledge of the latter. This would be equivalent to attempting to elucidate the mechanism of a chemical reaction without knowing the nature or composition of the reactants. These premature efforts have not contributed much to an understanding of the mechanism of oxidative phosphorylation although they have spawned endless speculation and a host of hypothetical intermediates. It is not my intention to cover this facet of the field of oxidative phosphorylation. I should like to restrict my comments on this subject to some of the more chemical developments which are outgrowths of studies on submitochondrial particles which still retain phosphorylative activity.

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ETP_H has been isolated in a form which will couple electron flow to phosphorylation only in presence of soluble factor (Linnane *et al.*, 1958; Linnane *et al.*, 1960). Whether this factor is present or not the rate of oxidation of succinate or DPNH is exactly the same and the components which undergo oxidoreduction are the same. This observation provides clear evidence that the electron transport process can be geared to phosphorylation by one or more devices and that the gearing does not modify in a gross sense the nature of the electron transport process. This is a most important observation since it sanctions the strategy of studying electron flow in particles which no longer can couple this flow to synthesis of ATP. That is to say, the mechanism of electron flow is independent of the coupling process.

Reference is often made in the literature to tightly coupled mitochondria (Lardy *et al.*, 1952) and one gains the impression from this phrase that there are two kinds of electron flow—one coupled to phosphorylation and the other not. If such were the case, the generalization stated above about the mechanism and nature of electron flow being the same whether the system is coupled or uncoupled could not be correct. Hatefi, Jurtshuk & Haavik in our laboratory (1961) have studied respiratory control (obligatory coupling) in heart mitochondria and they have found that in ETP_H there is no respiratory control of the oxidation of either succinate or DPNH, whereas in mitochondria there is respiratory control of DPNH linked substrates, but not of succinate. The one salient difference between ETP_H and mitochondria as far as the electron transport system is concerned is that free DPNH is involved with the former and bound DPNH with the latter. According to Hatefi, Jurtshuk & Haavik (1961), respiratory control is involved only at the step in which DPNH undergoes oxidation. That is to say, the oxidation of DPNH cannot go on unless inorganic phosphate is present, whereas in ETP_H the oxidation of free DPNH does not depend upon the presence of inorganic phosphate.

Under optimum conditions the P/O ratio for the oxidation of succinate is 2 for mitochondria (Lardy, 1955) and 1 for ETP_H (Linnane *et al.*, 1960); and for the oxidation of DPNH, 3 for mitochondria (Lardy, 1955) and 2 for ETP_H (cf. Table 13) (Linnane *et al.*, 1960). However, as discovered by Ziegler, Löw & Krueger in our laboratory (Ziegler *et al.*, 1961), these discrepancies disappear when measurements of the P/O ratio of ETP_H are carried out in presence of 5% CO_2 in air. In some as yet unexplained fashion CO_2 induces theoretical P/O ratios for the oxidation of both succinate and DPNH by ETP_H. Whatever the explanation, it is now possible to retain full phosphorylative capacity in a submitochondrial particle. In presence of CO_2, ETP_H shows respiratory control of the oxidation of DPNH: in

TABLE 14—P/O RATIOS FOR OXIDATION OF SUCCINATE AND DPNH BY $ETP_H \pm CO_2$

	$-CO_2$	$+CO_2$
P/O for succinate	1	2
P/O for DPNH	2	3
Respiratory control for succinate	0	0
Respiratory control for DPNH	0	+

Data of Ziegler, Löw & Krueger (Ziegler *et al.*, 1961).

absence of inorganic phosphate electron flow comes to a halt. It would appear from this observation of Ziegler, Löw & Krueger (1961) that ETP_H does not show respiratory control in absence of CO_2 since the particular oxidation reaction involved in this control is uncoupled. When coupling is restored by addition of CO_2 respiratory control is then restored *pari passu*.

Several submitochondrial particles with phosphorylative capacity have been described in the literature. Some are variations on the theme of ETP_H (e.g. a particle prepared by Racker & Pullman (Pullman *et al.*, 1960) by disintegration of a heavy beef heart mitochondrial fraction in a Mickle mill; and particles prepared by sonic oscillation of rat liver mitochondria by Kielley (Kielley *et al.*, 1958) and Lardy (McMurray *et al.*, 1958)). Lehninger and his group (Lehninger, 1955) have prepared a particle from rat liver mitochondria by treatment with digitonin and this catalyzes the coupled oxidation of β-hydroxybutyrate but not of succinate or DPNH (P/O of about 2).

When ETP_H is prepared in presence of EDTA, one or more factors essential for coupling are lost. Within the past year reports have come from the laboratories of Racker (Pullman *et al.*, 1960a, b) and Lehninger (Green *et al.*, 1959) and from the Enzyme Institute (Linnane *et al.*, 1960) on the isolation of coupling factors. One of these, according to Pullman *et al.*, has the properties of an ATPase (Pullman *et al.*, 1960b). Two factors isolated in Lehninger's laboratory have been implicated in the ADP–ADP exchange reaction and under appropriate conditions these factors can restore the phosphorylative activity of digitonin particles which have been treated in some appropriate fashion. All these observations add up to the conclusion that

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FIG. 15 (a). Membranous lamellae in chloroplast of *Chlamydomonas Moewusii*. Fixed in Kellenberger's buffered O_8O_4, stained with uranyl acetate, embedded in Epon. Siemens Elmiskop II B, × 100,000. (Courtesy of H. Ris.)

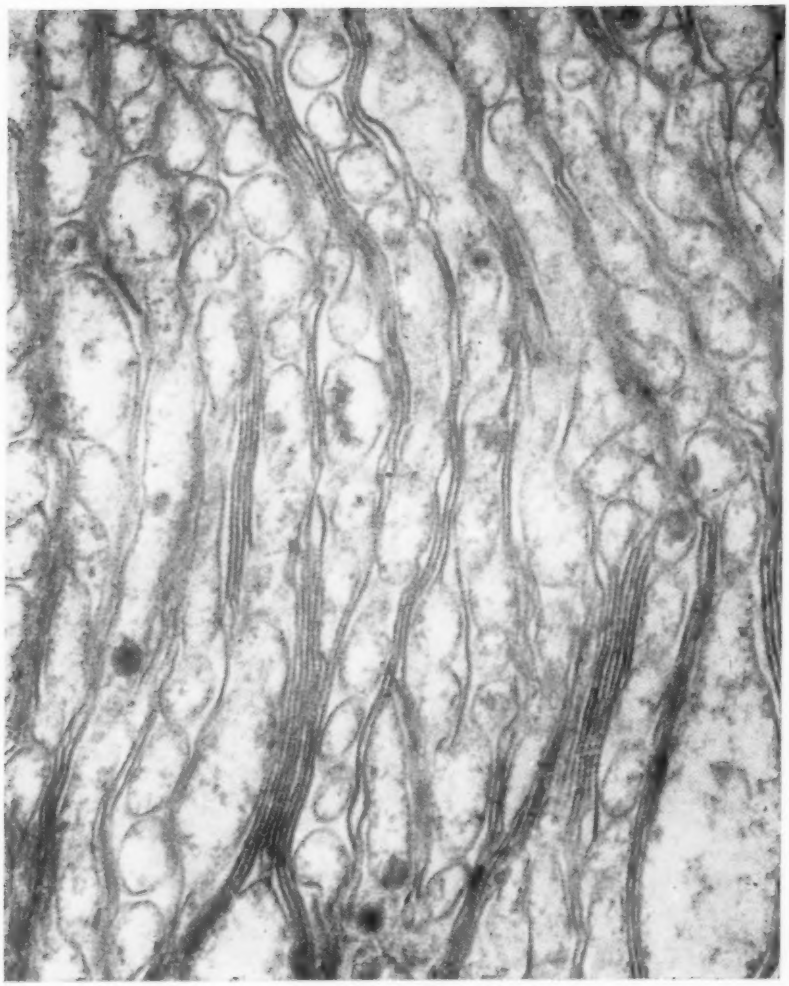


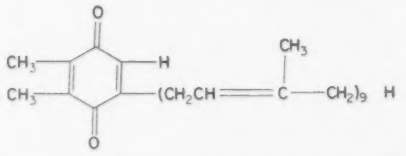
FIG. 15 (b). Membranous lamellae in chloroplast of *Antheros*. Details as in legend of Fig. 15 (a), × 80,000. (Courtesy of H. Ris.)

the conversion of the primary high energy bond to the pyrophosphate bond of ATP involves a multiplicity of transphosphorylation enzymes and factors and that these factors underlie the well-known phenomena of latent mitochondrial ATPase activity (Pressman *et al.*, 1956), the ATP–ADP exchange reaction (Wadkins *et al.*, 1958), and dinitrophenol-stimulated ATPase activity of phosphorylating particles (Lardy *et al.*, 1953; Slater, 1957). But it should be stressed that these factors are unrelated to the primary events of high energy bond formation which take place in the structured milieu of the electron transport chain. Rather are they concerned with the secondary stages by which the high energy bond is manipulated to lead to ATP formation. The primary process, which takes place in the electron transport chain, is the essence of oxidative phosphorylation; the secondary process, which proceeds outside the chain, is transphosphorylation or the transfer of a phosphate group from some as yet unknown compounds eventually to ADP.

As stated above, at the present moment there is no precise knowledge of the mechanism of oxidative phosphorylation though the literature abounds with speculations on this score. This is not the place to air these interesting speculations.

20. THE MITOCHONDRION VERSUS THE CHLOROPLAST

There have been spectacular developments in recent years in the field of photosynthesis and largely by virtue of the brilliant work of Arnon and his colleagues (Arnon *et al.*, 1959) it has been established that the chloroplast is a biochemical machine in plants and bacteria which couples the photochemical cleavage of water or other molecules to the synthesis of ATP. While the details are still vague it would appear that electrons originating from water are transferred through a chain involving cytochromes and other components and that this transfer is coupled to synthesis of ATP. This important discovery brings the chloroplast into line with the mitochondrion. The essential difference lies in the way in which electrons originate: from organic substrates by oxidation in the mitochondrion or from water by photolysis in the chloroplast. From then on the process is very similar in the two cases. It is of interest to point out in this context that Crane in our laboratory (Crane, 1959) has demonstrated that chloroplasts contain plastoquinone—a compound very similar to coenzyme-Q (see formula below).



Formula 2. Plastoquinone (Trenner *et al.*, 1959).

The chloroplast, like the mitochondrial, is rich in lipid (Frey-Wyssling, 1957) and electron microscopic studies (Hodge *et al.*, 1955; Steinman *et al.*, 1955) reveal structural arrangements not unlike those characteristic for mitochondria (cf. Fig. 15 (a), (b)).

21. LIFE-TIME AND ORIGIN OF MITOCHONDRIA

Until very recently there was no knowledge of the life span of a mitochondrion. A recent study by Sanadi (Fletcher *et al.*, 1961) provides the first estimate. According to his measurements on liver mitochondria the half-life is about 5–10 days. This means that the cellular synthesis of mitochondria is a continuing process and it is, therefore, appropriate to inquire by what sequence of events the mitochondrion is built up and how it is dismantled whether in part or in whole.

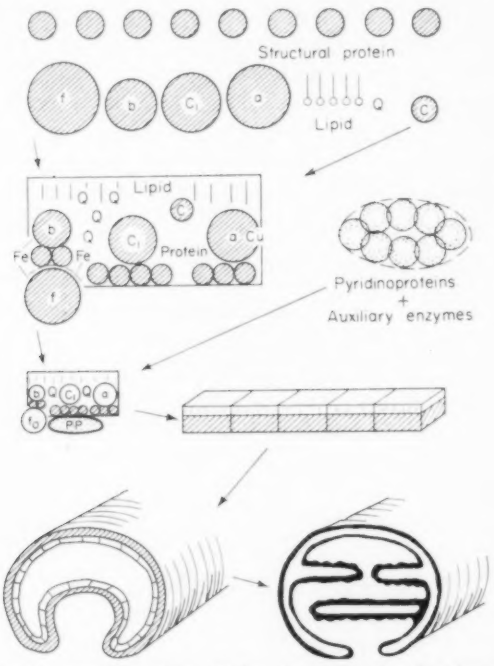


FIG. 16. Schematic representation of the stages in the development of a mitochondrion from the component building stones (flavoproteins, cytochromes, metals, lipid, auxiliary enzymes, etc.).

The biochemical evidence suggests that the mitochondrion can be synthesized or built up stepwise by virtue of the following processes which could proceed spontaneously: (1) the polymerization of monomers of the structural protein in presence of lipid and of the components of the electron transport chain to form the unit of the electron transport system; (2) the specific complexing of the auxiliary enzymes and coenzymes to form an aggregate which binds to the electron transport unit; (3) the formation of arrays of electron transport units to form a sheet with lipid between successive units: (4) the unfolding of the sheet to form external membrane and cristae; (5) the sequestering of ancillary enzymes within the intercristate space to specific binding sites on the structured electron transport

units (cf. Fig. 16). The biochemical evidence would suggest that the parts of the chain are constructed in such a fashion that under the proper conditions they can assemble to form the functional unit spontaneously just as the separated strands of the DNA molecule can recombine to form the original double-stranded molecule (Marmur *et al.*, 1960). Much the same would apply to the polymerization of electron transport units into a sheet and to the complex infoldings of this sheet necessary to form crista, intercristate spaces and external membrane. The tendency to polymerize and the guiding influence of highly specific binding sites would take care of the rest.

22. SOME CONCLUDING REMARKS

The key concepts which have thus emerged from the study of mitochondrial structure and function may be summarized as follows. The smallest common denominator of mitochondrial action has universal application in the same sense that nucleic acid is the universal of genetic determination. This common denominator is a rod-like capsule made up of twin electron transport units, the outer walls of which are made up of polymeric units of the structural protein and the interior of which is a phospholipid medium contributed by each of the two units. Within the phospholipid medium and attached to the structural protein are the fixed cytochromes, c_1 and a. Coenzyme-Q and cytochrome-c are the mobile components in the phospholipid medium which shuttle electrons between the fixed cytochromes. The oxidoreductions which lead to coupling take place within this protected or insulated phospholipid medium. An important aspect of this arrangement is the minimal reliance upon randomness. The transition from the three-banded capsular disposition of paired units in the mitochondrion or ETPH to the unpaired units of ETP is accompanied by the loss of the capacity for oxidative phosphorylation.

The mitochondrial electron transport chain can be stepwise resolved into each of five enzymatically active segments which could be in the form of particles or a soluble complex. These are: (1) the soluble complex of citric cycle enzymes; (2) the succinic-Q reductase complex; (3) the DPNH-Q reductase particle; (4) the QH_2-c reductase particle; and (5) the cytochrome-c oxidase particle. Under appropriate conditions the entire electron transport chain can be reconstructed by the recombination of the various segments.

Electron flow in the electron transport chain is localized in these five multicomponent complexes or particles which are involved respectively in the transfer of electrons from substrate to DPN, from succinate or DPNH to Q, from QH_2 to cytochrome-c and from reduced cytochrome-c to molecular oxygen. There is no precedence in classical chemistry for the mechanism of electron flow through these particles or complexes. It has been suggested by R. Bock and R. Criddle that the wave of sequential oxidoreductions which moves through the complex involves the pendulum action of one of the coenzymes which is bound to its apoprotein by an elastic link. The rotation of the coenzyme made possible by this elastic link permits of successive collisions with other functional groups in the complex.

As you may readily sense from my talk the field of mitochondrial structure and function is approaching a climactic point. Discoveries are being made with dizzying rapidity and it is not easy to make a fully documented presentation at this present moment. I have tried to give you the 1961 picture of the subject even though it will be subject to considerable amendment in the near future. But whatever the uncertainties we can be sure on one point, namely that the mechanism of oxidative phosphorylation cannot elude us much longer.

The tactic of successive approximations is almost mandatory for studying a problem as broad as that of the mitochondrion. If an effort is to be made to deal with the problem as a whole then it becomes necessary to deal with enzymological, electron microscopic, biophysical, biological and chemical parameters simultaneously. Depth of understanding in just one dimension is not only difficult to achieve but relatively useless since one is dealing with an interrelated whole. The mitochondrion has served as a model for working out the tactics of solving the problem of a biochemical machine and these tactics may now be applied to the study of other as yet unexplored biochemical machines.

In concluding may I pay a tribute to some of the investigators whose contributions have been invaluable for the development of our knowledge of mitochondrial structure and function. O. Warburg was the first to recognize the association of terminal respiration with particles and the influence of his work on those engaged in the study of electron transport has been profound. The discovery and concept of the electron transport and cytochrome chain is the great contribution of D. Keilin and all the work that has been discussed in this lecture is only an extension of his classical studies. H. Theorell and his school defined with considerable precision the chemical and physical characteristics of the flavoprotein and cytochrome enzymes and these basic studies have served as the foundation stones for much of the more recent developments. Britton Chance is the youngest of this set of greats whose contribution to the foundations of mitochondrial structure and function has been decisive. He and his school have refined the tool of rapid flow spectrophotometry to the point where it was possible to recognize the main absorbing species in the electron transport chain and to determine quantitatively both their concentrations and their turnover rates.

The phenomenon of oxidative phosphorylation which was discovered independently by H. Kalekar and V. Belitzer has been intensively studied at the mitochondrial level by a distinguished group of investigators, notably Ernster, Hunter, Lardy, Lindberg, Lehninger, Ochoa and Slater. They have defined the precise values of the P/O ratios for various substrates, the behavior of the phosphorylating system in media of varying osmotic concentrations, the factors which stabilize and lead to deterioration of phosphorylation, the inhibitors which specifically affect the phosphorylative process and they have developed the concepts of latent ATPase activity and respiratory control.

The idea that the enzymes of the citric acid cycle as well as those connected with fatty acid oxidation, electron transport and oxidative phosphorylation are all part of one giant complex—an idea which was the basis of the cyclophorase

hypothesis first advanced by our laboratory in 1947 (Green *et al.*, 1948) became generally accepted largely owing to the important demonstration in 1948 by Lehninger and Kennedy (Lehninger *et al.*, 1948) and independently by Schneider and Potter (Schneider *et al.*, 1949) in 1949 that the mitochondrion was in fact the physical housing of the cyclophorase complex of enzymes. The introduction of the technique of differential centrifugation of particles in sucrose solutions by Claude, Hogeboom & Schneider (Claude, 1947; Hogeboom *et al.*, 1948) facilitated the isolation of purified preparations of mitochondria, and opened the door to the systematic, biochemical study of mitochondria.

In the area of the Krebs citric cycle enzymes, Massey, Reed, Schweet and Sanadi have added much to our knowledge of the mechanisms by which pyruvate and *a*-ketoglutarate are oxidatively decarboxylated and of the cofactors which participate in these multienzyme sequences.

Finally one must pay a tribute to the work of Stotz and Marinetti on mitochondrial lipids, of Wainio, Ball and Stotz on the fractionation of particles and of Boyer and Cohn on the exchange reactions related to oxidative phosphorylation.

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ENZYMATIC SYNTHESIS OF FATTY ACIDS

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Available evidence indicates that there are two distinct systems for the synthesis of fatty acids. 1—The mitochondrial system which may involve some enzymes of the β-oxidation system plus TPNH-α-β-unsaturated acyl reductase and perhaps a new condensing enzyme. Both TPNH and DPNH are required for the synthesis. This system is for the elongation of fatty acids and is responsible for the formation of stearate from palmitate and arachidonate from lionoleate, etc. 2—The non-mitochondrial system which is located in the cytoplasm and catalyzes the conversion of acetyl CoA to palmitate in the presence of ATP, Mn^{2+}, HCO_3^- and TPNH. Acetyl CoA is condensed with HCO_3^- to form malonyl CoA, a reaction which is catalyzed by acetyl CoA carboxylase (a biotin-containing enzyme) in the presence of ATP and Mn^{2+}. Malonyl CoA condenses with acetyl CoA or propionyl CoA to yield a reduction by TPNH saturated fatty acids, CO_2 and CoA. Short-chain fatty acyl CoA's (C_4, C_6, C_8, etc.) did not accumulate suggesting they may not be intermediates (or in equilibrium with such intermediates) in the overall synthesis. Instead a compound (presumably not acetoacetate) was isolated from the condensation of malonyl CoA and acetyl CoA which could be converted to palmitate by the addition of TPNH to the reaction mixture.

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FOR many years one of the predominating concepts in biochemistry has been the general assumption that synthetic and degradative processes are alternate aspects of a reversible process. This thesis was widely accepted and was thought to include processes involved in the synthesis of proteins, carbohydrates, fats, etc. However, this hypothesis has lost most of its attractiveness and glamour: in actuality every instance in which this was thought to apply, experimental results proved the contrary. Fatty acid synthesis was no exception. Until two years ago, the predominant concept of the mechanism of fatty acid synthesis was that it occurred via the reversal of the enzymatic reactions involved in β-oxidation (Wakil *et al.*, 1957; Porter *et al.*, 1957; Porter & Tietz, 1957; Stumpf & Barber, 1956; Gibson *et al.*, 1958a, b; Wakil *et al.*, 1958; Squires *et al.*, 1958; Wakil, 1958; Wakil & Ganguly, 1959; Brady, 1958; Lynen, 1959). This concept was advocated by Lynen early in 1953, and generally accepted by the biochemical community despite earlier observations by Gurin and his colleagues (Brady & Gurin, 1952; Dituri & Gurin, 1953; Van Baalen & Gurin, 1953) on the possible presence of two separate and distinct systems for synthetic and degradative processes.

Experimental evidence supporting fatty acid synthesis via "a modified scheme for reversal of β-oxidation sequence" came from two independent observations. The first was the discovery by Langdon (1957) that crotonyl CoA can be reduced by TPNH in the presence of an enzyme found in the soluble extracts of rat liver; the second was the demonstration by Stumpf & Barber (1957) and by Wakil *et al.* (in press) that stearic acid synthesis from acetyl CoA and palmityl CoA was catalyzed by mitochondrial enzymes in the presence of DPNH and TPNH.

Pyridoxal phosphate (Wakil, in press) appears to be a co-factor in this conversion, a fact which is supported by nutritional studies relating to the *in vivo* synthesis of long-chain fatty acids. Since the aforementioned system is located in the mitochondria and is concerned primarily with the elongation of fatty acids of moderate chain length, the name "mitochondrial" or "elongation" system for fatty acid synthesis has been proposed for this series of reactions.

Evidence for a second pathway of fatty acid synthesis was obtained at the Enzyme Institute at the University of Wisconsin (Gibson *et al.*, 1958a; Wakil *et al.*, 1958; Gibson *et al.*, 1958b; Wakil, 1958; Wakil & Ganguly, 1959) by the isolation of a new enzyme system from avian liver that was free of the key enzymes of the β-oxidation sequence. This system converted acetyl CoA to long-chain fatty acids in the presence of ATP, Mn^{2+}, CO_2 and TPNH. It was not associated with mitochondria nor with microsomes but it appeared to be associated with particles of a size smaller than microsomes (the whole system may be separated as a pellet on centrifugation at $140{,}000 \times g$ for 2 hr). Furthermore, malonyl CoA is the key intermediate (Wakil, 1958; Wakil & Ganguly, 1959) in this system and is the compound which contributes all but one of the C_2 units going into the final acyl CoA product. For these reasons, the name "non-mitochondrial system" or "malonyl CoA pathway" for fatty acid synthesis has been proposed for this system.

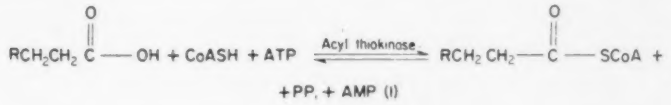
THE "MITOCHONDRIAL SYSTEM" FOR FATTY ACID SYNTHESIS

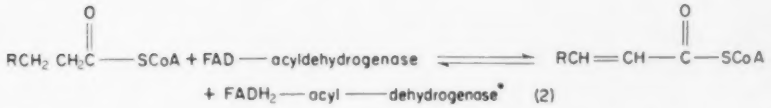
In 1953, several laboratories (Green, 1954; Lynen & Ochoa, 1953; Drysdale & Lardy, 1953; Lehninger & Greville, 1953) announced the elucidation of the mechanism of fatty acid oxidation, demonstrating that fatty acids are oxidized via a pathway very similar to the β-oxidation scheme proposed by Knöop (1904) 50 years earlier. The sequence of the reactions and the enzymes involved are given on the following page.

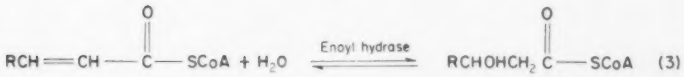
Each of the enzymes involved in this sequence of reactions has been prepared in a highly purified state and each step has been shown to be reversible. The equilibrium constants for the first four reactions were shown to be near unity whereas the thiolase reaction has an equilibrium constant of $1{\cdot}6 \times 10^{-5}$ at pH 7·0 (Lynen & Decker, 1957), this means that in the presence of CoASH there is very little β-ketoacyl CoA present. Nevertheless, the equilibrium can be shifted toward condensation in the presence of DPNH and β-hydroxyacyl dehydrogenase.

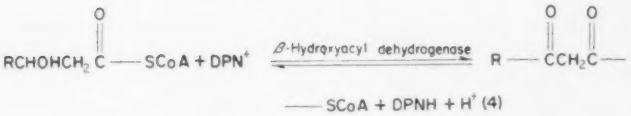
Stadtman & Barker (1949a, b, c, d, e; 1950; Barker, 1956) were the first to demonstrate the conversion of labeled acetate into short-chain fatty acids by water-soluble enzyme preparation which they obtained from *Clostridium Kluyveri*. Short-chain fatty acids (butyrate and hexanoate) were synthesized by the *Kluyveri* extract and the reactions from acetyl CoA to butyryl CoA and hexanoyl CoA were catalyzed presumably by the enzymes of the β-oxidation sequence. As yet no reconstitution experiments of the fatty acid synthesizing system from *Kluyveri* have been reported; neither is there any definite information to the principal pathway of fatty acid synthesis in the organism. Recently Vagelos & Alberts (1960)

have shown that extract of *Cl. Kluyveri* catalyzed an exchange reaction between malonyl CoA and CO_2 which was completely dependent upon the presence of acyl CoA. The role and significance of this enzyme in fatty acid synthesis have to await further information.









Stansly & Beinert (1953) demonstrated the conversion of acetyl CoA to butyryl CoA in the presence of DPNH, a reduced dye, and the purified enzymes of the fatty acid oxidation cycle. They were unable to demonstrate the formation of significant amounts of longer acyl CoA derivatives. This appeared to be due to the preferential condensation of acetyl CoA with another molecule of acetyl CoA rather than with higher acyl CoA homologs (buturyl CoA, hexanoyl CoA, etc.).

* The reduced acyldehydrogenase is then oxidized by the electron transferring flavoprotein to an FAD-acyl dehydrogenase and reduced electron transferring flavoprotein (Crane *et al.*, 1956), the latter enzyme then transfers its electron to a suitable acceptor (cytochrome *c* or a dye).

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A noted weakness in these experiments was the requirement of reduced dye as an electron donor rather than a natural electron donor such as reduced pyridine nucleotides, reduced flavins, reduced cytochromes, etc.

Langdon (1955, 1957) discovered an enzyme (TPNH crotonyl CoA reductase) in soluble extracts of rat liver that catalyzed the reduction of crotonyl CoA by TPNH. Seubert *et al.* (1957) isolated this enzyme from pig liver mitochondria and purified it extensively by differential centrifugation in the presence of cholate. The purified particulate enzyme was free from the various enzymes of the β-oxidation cycle and had a wide range of specificity, ranging from crotonyl CoA to α-β-unsaturated stearyl CoA. The localization of this enzyme in the cell is not yet certain. Seubert and his colleagues claimed it to be associated with mitochondria while Popjak and his associates (Lachance *et al.*, 1958) claimed to have been able to isolate it from microsomes. With the aid of this enzyme, Seubert *et al.* (1957) were able to reconstitute a fatty acid synthesizing system from the purified enzymes of the β-oxidation cycle (thiolase, enoyl hydrase and β-hydroxyacyl dehydrogenase), a source of DPNH (alcohol and alcohol dehydrogenase) and a source of TPNH (glucose-6-phosphate and glucose-6-phosphate dehydrogenase). With this system they were able to demonstrate the synthesis of octanoyl and capryl CoA from hexanoyl CoA and acetyl-1-^{14}C CoA.

Stumpf & Barber (1957) were the first to show fatty acid synthesis in intact mitochondria derived from avocado mesocarp. Adenosine triphosphate, CoA and Mn^{2+} were essential components for the conversion of ^{14}C acetate into the long-chain fatty acids (palmitic and oleic acids) in this system.

Recently, Wakil *et al.* (in press; Wakil, in press) were able to demonstrate that intact mitochondria isolated from pigeon, rat or beef livers can synthesize long-chain fatty acids from acetyl CoA (cf. Table 1). Mitochondria were prepared in either 0·25 M or 0·88 M sucrose according to the general procedures of Hogeboom *et al.* (1948). When the mitochondria were incubated anaerobically with acetyl-1-^{14}C CoA, ATP, DPNH and TPNH, ^{14}C-labeled long-chain fatty acids were isolated. Under these conditions the conversion of acetyl CoA to fatty acids was completely dependent upon and directly related to the presence of ATP (cf. Fig. 1). In the presence of 10 μmole of ATP, 10–20 per cent of the acetyl CoA was converted to fatty acids. Neither buturyl CoA nor GTP could substitute for ATP whereas ADP, CTP and UTP could replace ATP at relatively high levels.

The incorporation of acetyl CoA into the fatty acids was proportional to the amounts of mitochondria added as shown in Fig. 2. Anaerobic conditions were necessary in order to minimize the oxidation of DPNH and TPNH by molecular oxygen. Both reduced pyridine nucleotides (TPNH and DPNH) were required for optimum synthesis: in the absence of either reduced nucleotide there was 60 to 80 per cent inhibition of synthesis, while in the absence of both nucleotides there was no synthesis at all.

Bicarbonate was not required for fatty acid synthesis in this system. This is in contrast to the non-mitochondrial system which is absolutely HCO_3^- dependent. Furthermore, ^{14}C-malonyl CoA was incorporated into the fatty acids only in the

presence of ATP (cf. Table 1). Malonyl CoA is known to be decarboxylated to acetyl CoA by a mitochondrial enzyme (Nakada *et al.*, 1957) and it seems possible that this accounts for the utilization of malonyl CoA in the presence of ATP.

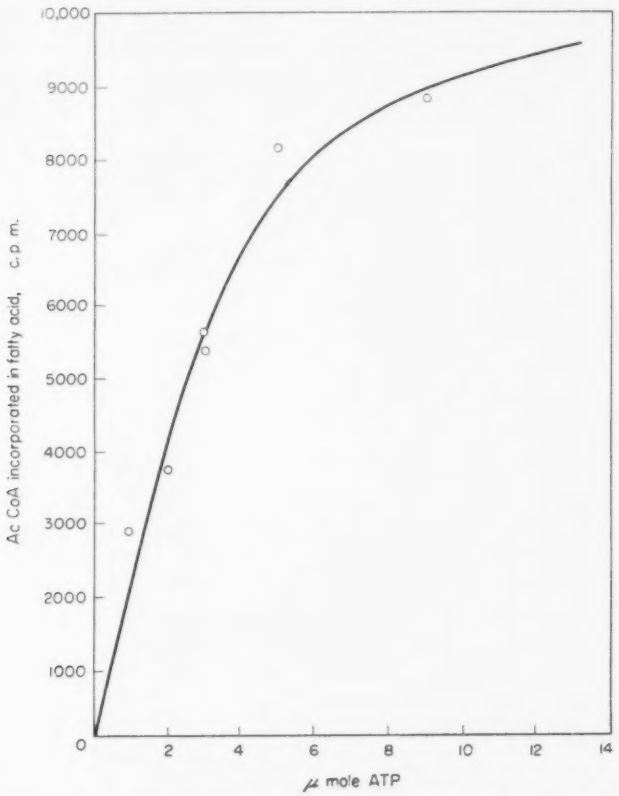


FIG. 1. ATP requirement in fatty acid synthesis by mitochondria: the reaction mixtures contained 34·5 mμmole of AcCoA (55,200 c.p.m.), 0·25 μmole TPNH, 0·25 μmole DPNH, 2·8 mg of mitochondrial protein, 50 μmole phosphate (pH 6·5), ATP as indicated and H_2O to a final volume of 0·5 ml. The reaction mixture was incubated anaerobically at 38° for 1 hr.

The products from the incorporation of acetyl-1-^{14}C into fatty acids by mitochondria were separated from the reaction mixture by the procedure of Wakil *et al.* (1957) and were identified by a variety of techniques (Reid & Ledener, 1951; Kaufmann & Nitsch, 1954). Fig. 3 shows the distribution of the radioactivity among the various acids (C_{12} to C_{20}) when separated by reverse-phase chromatography according to the procedure of Kaufmann & Nitsch (1954). The major component of the synthesized fatty acids was stearic acid (40 per cent) with arachidate, palmitate, myristate and laurate constituting the remainder.

Stearic acid synthesized from 1-^{14}C-acetyl CoA was isolated by dilution with unlabeled stearic acid, recrystallized from various solvents to a constant specific activity, and the resulting acid decarboxylated by the Schmidt reaction as described

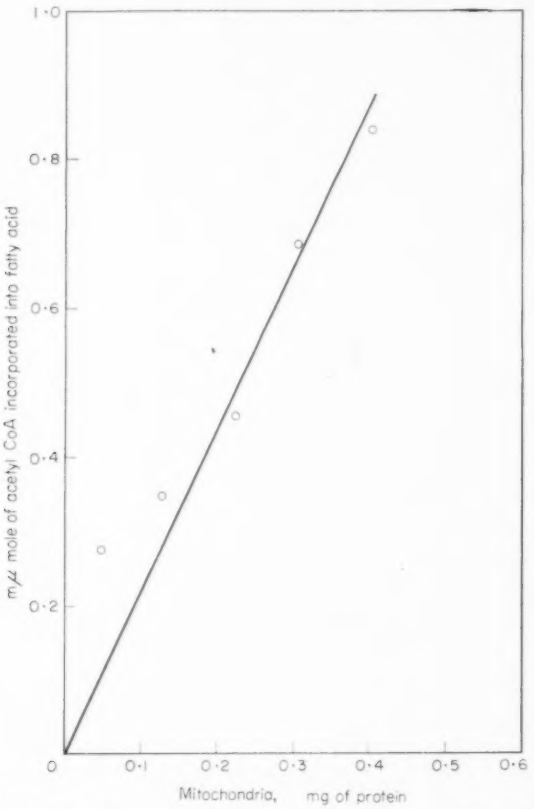


FIG. 2. The relationship between ^{14}C-acetyl CoA incorporation in fatty acids and the amount of mitochondria: each reaction mixture contained 58·6 mμmole 1-^{14}C-acetyl CoA (152,000 c.p.m.), 0·1 μmole TPNH, 0·1 μmole DPNH, 2 μmole ATP, 30 μmole phosphate buffer (pH 6·5) and water to a final volume of 0·5 ml. The reaction was started with the addition of freshly prepared rat liver mitochondria as indicated. The reaction mixture was incubated anaerobically at 38° for 1 hr.

by Phares (1951). The specific activity (c.p.m. per μatom carbon) of the liberated CO_2 was more than twice the specific activity of stearic (expressed as c.p.m. per μatom carbon), indicating an elongation of a pre-existing short-chain fatty acid by the successive additions of acetyl CoA. This is in contrast with the synthesis of palmitate from acetyl CoA by the non-mitochondrial system which is true *de novo* synthesis (Porter & Tietz, 1957).

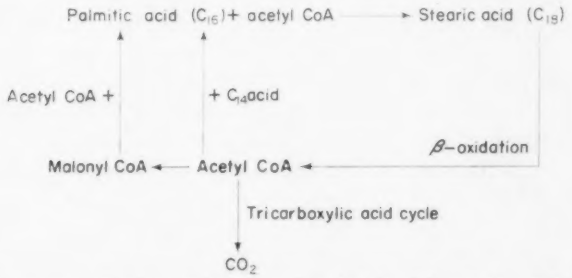
These results are in complete agreement with reported experimental data on the synthesis of stearate and palmitate in whole animals (Smedley-Maclean & Hoffer, 1926; Sonderhoff & Thomas, 1937; Schoenheimer & Rittenberg, 1937; De Stetten

TABLE 1—FATTY ACID SYNTHESIS BY MITOCHONDRIA

The reaction mixture contained 34·5 mμmole of AcCoA (55,200 c.p.m.) 8 μmole ATP, 0·25 μmole TPNH, 0·25 μmole DPNH, 2·8 mg mitochondrial protein, 50 μmole phosphate, (pH 6·5) and H_2O to a total volume of 0·5 ml. 4·0 μmole $KHCO_3$ and 30 μmole malonyl CoA ($HOOCCH_2$ $^{14}COCoA$ 70,000 c.p.m.) were used where indicated. The reaction mixture was incubated anaerobically at 38° for 1 hr.

	AcCoA incorporated into long-chain fatty acids (mμmole)
Complete system	4·5
No ATP	0·3
No TPNH	2·4
No DPNH	2·0
No TPNH, No DPNH	0·0
Complete system + HCO_3^-	3·0
No acetyl CoA + Malonyl CoA	2·4
No acetyl CoA + Malonyl CoA, without ATP	0·8

& Schoenheimer, 1940; Klein, 1943; Rittenberg & Bioch, 1944, 1945; Stevens & Chaikoff, 1951; Zabin, 1951; Anker, 1952; Dauben *et al.*, 1953; Conigliso & Cate, 1958). The significant finding by many investigators was that palmitic acid (C_{16}) can be converted to stearic acid (C_{18}) by the addition of one C_2-unit while the reverse does not appear to be possible. In other words, the conversion of stearic acid to palmitate is not achieved by the mere cleavage of one C_2-unit but instead is brought about by the complete oxidation of stearic acid to acetyl CoA and the subsequent conversion of acetyl CoA to palmitic acid via either the malonyl CoA pathway or by condensation on a pre-existing myristic acid (C_{14}). This is illustrated by Scheme I.



SCHEME I. Relationship between palmitic and stearic acids.

The inability of stearic acid to be directly converted to palmitic acid by the loss of one C_2-unit may be explained by the well-known observation that a fatty acid molecule being oxidized by the β-oxidation enzymes (mitochondria or whole animal systems) is completely converted to acetyl CoA with very little if any chance of leaving the enzyme prior to complete oxidation (Green, 1954; Kennedy & Lehninger, 1952; Lardy & Wellman, 1952; Judah & Ress, 1953). Thus, once stearic acid is on the path of oxidation it continues until it is completely converted to acetyl CoA. The resulting acetyl CoA can be either oxidized to CO_2 by the tricarboxylic acid cycle (TCA cycle) or can be channeled into the various synthetic reactions, e.g. palmitic acid, amino acids, carbohydrates, purines, etc. Palmityl CoA is synthesized primarily by the non-mitochondrial system (the malonyl pathway) and can be elongated by the mitochondria by the addition of acetyl CoA to form stearyl CoA. On this basis, therefore, the addition of ^{14}C-1-acetyl CoA would result in the formation of stearic acid labeled predominantly in the carboxyl group of the molecule. This, indeed, appeared to be the case in the stearic acid synthesized by rat liver slices as reported by Zabin (1951) who found that the ^{14}C content of the carboxyl carbon of stearic acid derived from acetate-1-^{14}C was higher than the rest of the carbon atom of the molecule, while the remaining ^{14}C in carbon atoms 3 to 18 of stearic acid appeared to be uniformly distributed along the chain. On the other hand, palmitic acid which was isolated simultaneously from the liver slices (Zabin, 1951) showed isotope concentration uniformly distributed along the entire molecule. These results suggest that the addition of the last two carbon atoms to palmitate (C_{16}) to form stearate (C_{18}) is accomplished by different mechanism than the multiple condensation of the C_2 units to form the C_{16} acid. The mitochondrial system appears to be concerned with such elongation of C_{16} acid to form C_{18} acid and may account for the variation of the isotope ratio in carbons 1 and 2 of C_{18} acid as compared to the remaining carbon atoms.

Other investigators, Anker (1952), Dauben *et al.* (1953) and Conigliso & Cate (1958) arrived at similar conclusions after *in vivo* experiments with rats and mice, using ^{14}C labeled acetate and myristate. Their results and conclusions are compatible with the aforementioned concept, namely, the synthesis of palmitic and myristic acids is accomplished by multiple condensation of C_2-units, resulting in a labeling throughout the molecules, while the C_{18} acids (stearic, etc.) are labeled significantly higher in the carboxyl end than the remaining carbon atoms of the molecule.

The incorporation of acetyl CoA into C_{20} fatty acids (cf. Fig. 3) is extremely significant and may indicate that the mitochondrial system is the site for synthesis of these acids by the addition of one acetyl CoA unit onto a C_{18} fatty acid. When stearyl CoA was incubated anaerobically with 1-^{14}C-acetyl CoA, mitochondria, TPNH and DPNH, the long-chain fatty acids were isolated and tentatively identified as a C_{20} fatty acid (possibly arachidic acid) by paper chromatography. Similar condensation of 1-^{14}C-acetyl CoA can be demonstrated with oleyl CoA as substrate instead of stearyl CoA and the formation of C_{20} acid (cf. Table 2).

We have not as yet identified the C_{20} acid produced from either stearyl CoA or oleyl CoA, neither have we any information as to whether the C_{20} produced from oleyl CoA still retains its double bonds. If the C_{20} acid derived from oleyl CoA is

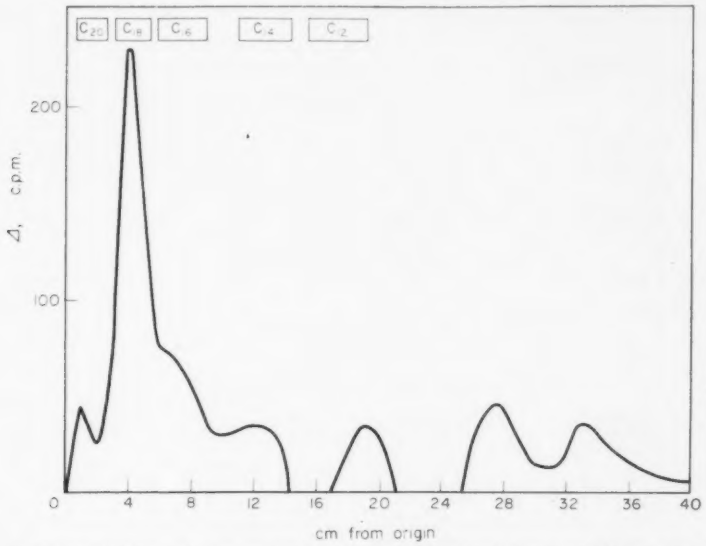


FIG. 3. Distribution of the mitochondria synthesized fatty acids in the paper chromatographic system of Kaufmann & Nitsch (1954).

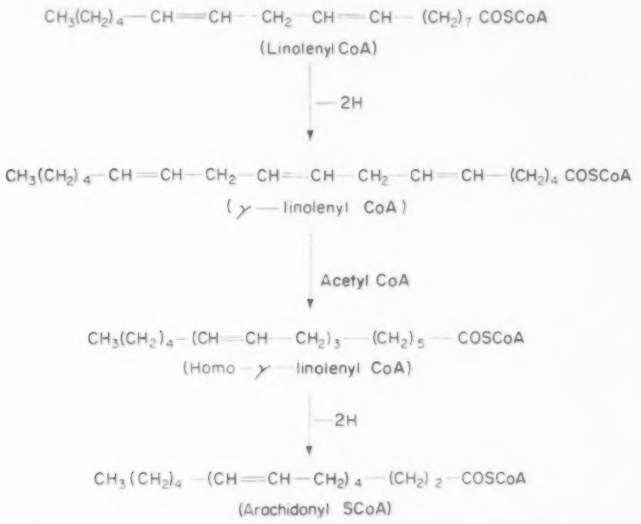
TABLE 2—ELONGATION OF THE FATTY ACYL CoA BY THE ADDITION OF ^{14}C-ACETYL CoA

Acyl CoA added	Fatty acid synthesized	mμmole AcCoA into the fatty acid
None	—	0·1
Myristyl CoA	C_{16}	4·5
Palmityl CoA	C_{18}	3·4
Stearyl CoA	C_{20}	2·0
Oleyl CoA	C_{20}	3·8

The reaction mixture contained 30μmole potassium phosphate (pH 6·5), 36 mμmole acety-l-^{14}C CoA (160,000 c.p.m.), 0·1 μmole DPNH, 0·1 μmole TPNH, 30 mμmole of the indicated acyl CoA and 0·5 mg of rat liver mitochondria in a final volume of 0·5 ml. The reaction mixture was incubated anaerobically for 1 hr at 38°.

unsaturated and if other unsaturated fatty acyl CoAs (linolyl CoA, linolenyl CoA) can substitute for oleyl CoA in this system, then the *in vivo* experiments of Mead and his colleagues (Mead *et al.*, 1953; Steinberg *et al.*, 1956, 1957; Mead, 1957; Mead & Howton, 1957) on the synthesis of arachidonic acid from ^{14}C-labeled stearic, linoleic and linolenic acids would be in accordance with this hypothesis.

According to Mead, linoleic acid is dehydrogenated to γ-linolenic acid which then adds acetyl CoA to form homolinolenic acid; the latter acid is subsequently dehydrogenated to arachidonic acid. The overall scheme proposed by Mead & Howton is as follows (CoA derivatives of these acids are probably the forms participating in these reactions):



SCHEME II. Possible pathway for the conversion of linoleyl CoA to arachidonyl CoA.

Elongation of fatty acids by soluble extracts of mitochondria

Soluble enzyme preparations of liver mitochondria were prepared by extracting the acetone powder of mitochondria with phosphate buffer (Wakil *et al.*, in press). Such extract can readily incorporate ^{14}C-acetyl CoA into higher-chain fatty acids in the presence of ATP, DPNH, and TPNH. The requirements for these components were absolute, which is in agreement with the results obtained from intact mitochondria. The product of synthesis in the soluble system was essentially similar to that obtained by whole mitochondria except that there was more incorporation in the relatively shorter-chain acids compared to the higher-chain acids.

Dialysis of the soluble extracts and their subsequent treatment with charcoal resulted in a sharp decline in enzymatic activity as shown in Table 3. Addition of the boiled extract of the enzyme preparation completely restored the enzymatic activity. Furthermore, the boiled extract could be completely replaced by two distinct compounds; an intermediate-chain fatty acid (C_8, C_{10}, C_{12}, C_{14}, etc.) and pyridoxal phosphate (cf. Table 3).

The requirement for the short-chain fatty acyl CoA indicated that this system causes the elongation of fatty acids by the addition of an acetyl CoA. Thus, when octanoyl CoA (C_8CoA) was used as an acceptor we were able to isolate C_{10} acid,

TABLE 3—FACTORS AFFECTING FATTY ACID SYNTHESIS BY SOLUBLE MITOCHONDRIAL EXTRACTS

Experiment no.	Additions	AcCoA incorporated into fatty acids (mμmole)
I	None	0·00
	ATP	0·29
	ATP and octanoic acid	3·00
	ATP and pyridoxal phosphate	0·79
	ATP and octanoic acid and pyridoxal phosphate	4·50
	ATP and octanoic acid and pyridoxamine phosphate	4·50
II	None	0·00
	ATP	0·20
	ATP and octanoic acid	1·88
	ATP and octanoic acid and pyridoxal phosphate	3·54
	ATP and octanoic acid and pyridoxamine phosphate	3·16
III	ATP	0·2
	ATP and pyridoxal phosphate	0·6
	ATP and boiled enzyme extract	1·3
	ATP and boiled enzyme extract and pyridoxal phosphate	2·61
	ATP and boiled enzyme extract and pyridoxamine phosphate	2·28
	ATP and boiled enzyme extract and pyridoxine HCl	1·5
	ATP and boiled enzyme extract and pyridoxamine HCl	1·88
IV	None	0·08
	Palmityl CoA	1·30
	Palmityl CoA pyridoxal phosphate	2·00
V	Palmityl CoA plus ATP	1·0
	Palmityl CoA plus ATP and pyridoxal phosphate	1·6

The reaction mixture contained 30 μmole of phosphate buffer, pH 6·5, 58 mμmole of 1-^{14}C-acetyl CoA (152,000 c.p.m.), 0·125 μmole DPNH, 0·125 μmole TPNH and water to a final volume of 0·5 ml. Where indicated the following were added: 2 μmole of ATP, 25 mμmole octanoic acid, 50 mμmole pyridoxal phosphate, 150 mμmole pyridoxamine phosphate, 150 mμmole pyridoxine HCl, 150 mμmole pyridoxamine HCl, 10 mμmole palmityl CoA and 0·05 ml of boiled enzyme extract (prepared by boiling the soluble extract of mitochondria for 5 min at 100°).

Five different enzyme preparations were added as follows: in experiment I, 0·13 mg of charcoal treated soluble extracts of beef liver mitochondria; in experiment II, 0·2 mg of charcoal treated soluble extracts of beef liver mitochondria; in experiment III, 0·62 mg of soluble extract of beef liver mitochondria; in experiment IV, 0·55 mg of soluble extract after sonic irradiation rat liver mitochondria and in experiment V, 1·5 mg of mitochondria after sonic irradiation.

All tubes were incubated under N_2 gas for 1 hr at 38°.

when C_{10} acid was used a C_{12} acid was isolated and so on. The higher fatty acids isolated in these experiments (i.e. C_{10} in the first example and C_{12} in the second) contained over 80 per cent of the incorporated ^{14}C-acetyl CoA.

When the corresponding acyl CoA derivatives (C_8CoA, $C_{10}CoA$, $C_{12}CoA$, etc.) were used, ATP was no longer necessary (cf. Table 3), demonstrating that the ATP was required for the formation of the acyl CoA's of these endogenous fatty acids. Butyryl CoA and hexanoyl CoA are very poor replacements for the higher-chain acyl CoA's in this system, indicating a specificity for the elongation of intermediate and long-chain fatty acids but not for the shorter-chain acids. This may be the result of either the lack of an enzyme that would catalyze the condensation of acetyl CoA with butyryl CoA and hexanoyl CoA or to the absence of the specific TPNH-α-β-unsaturated acyl CoA reductase for these fatty acids (Langdon, 1957; Wakil *et al.*, in press). Indeed, we could not detect the TPNH-crotonyl CoA reductase when the extract was assayed with crotonyl CoA as substrate (Langdon, 1957; Wakil *et al.*, in press). (We have not attempted as yet an assay for the reduction of longer-chain α-β-unsaturated acyl CoA derivatives by TPNH in the soluble extract.)

The requirement for DPNH and TPNH may be explained by the need for DPNH to reduce the condensation product (β-ketoacyl CoA), while TPNH is needed for the reduction of the α-β-unsaturated acyl CoA to the saturated derivative. The overall scheme may be written as follows, illustrating the conversion of palmityl CoA to stearyl CoA.

$$\text{palmityl CoA } (C_{16}CoA) + \text{acetyl CoA} \xrightarrow{\text{I}} \beta\text{-ketostearyl CoA} + \text{CoA} \qquad (6)$$

$$\beta\text{-ketostearyl CoA} + \text{DPNH} + H^+ \xrightarrow{\text{II}} \beta\text{-hydroxystearyl CoA} + \text{DPN}^+ \qquad (7)$$

$$\beta\text{-hydroxystearyl CoA} \xrightarrow{\text{III}} \alpha\text{-}\beta\text{-unsaturated stearyl CoA} + H_2O \qquad (8)$$

$$\alpha\text{-}\beta\text{-unsaturated stearyl CoA} + \text{TPNH} + H^+ \xrightarrow{\text{IV}} \text{TPN}^+ + \text{stearyl CoA} \qquad (9)$$

I = condensing enzyme possibly containing pyridoxal phosphate
II = β-hydroxyacyl CoA dehydrogenase
III = enoyl hydrase
IV = α-β-unsaturated acyl CoA reductase

The role of pyridoxal phosphate in fatty acid synthesis

Birch & György in 1936 found that rats suffering from vitamin B_6 deficiency developed symptoms of acrodynia similar to those obtained earlier by Burr & Burr (1929, 1930; Burr G. O. *et al.*, 1932; Burr G. O. 1942) with fatty acid-deficient rats; they also observed that unsaturated fatty acids exercised a sparing effect on vitamin B_6. These initial observations were confirmed by many workers and were extended to show that the acrodynia caused by pyridoxine deficiency can be overcome by feeding arachidonic acid, or linoleic acid (Birch & György, 1936; Sherman *et al.*, 1950). Sherman *et al.* were able to demonstrate an increase in the synthesis of arachidonic acid in rats receiving pyridoxine. Whitten & Holman

(1952) submitted evidence to show that rats fed on fat-deficient, pyridoxine-deficient diet developed acrodynia which can be relieved best by pyridoxine plus linoleate suggesting that pyridoxine-deficient animals may suffer a secondary deficiency of arachidonic acid.

Preliminary observations in our laboratory indicate that the relationship between pyridoxine and essential fatty acid metabolism may be confined to the role of pyridoxal phosphate in the elongation of fatty acids. When soluble extracts of the mitochondrial system were dialyzed and treated with charcoal, a partial but not complete requirement for pyridoxal phosphate was noted (cf. Table 3). The partial effect of pyridoxal phosphate was demonstrated not only in soluble extracts but also in particles derived from mitochondria. A two to threefold increase in the amount of ^{14}C-acetyl CoA incorporated into the longer-chain fatty acids was observed when the pyridoxal phosphate concentration in the reaction mixture was about 10^{-3} M. Pyridoxamine phosphate could substitute for pyridoxal phosphate, whereas pyridoxine or pyridoxal hydrochloride was ineffective (cf. Table 3). The response to added pyridoxal phosphate could be demonstrated in some but not in all preparations.

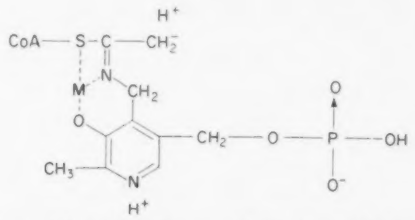
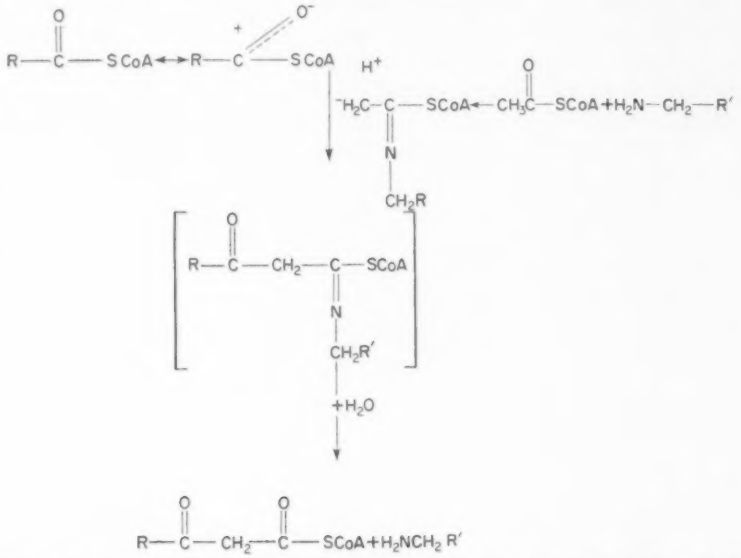


FIG. 4. A proposed model system for acetyl CoA, pyridoxamine phosphate and metal (M).

The precise role of pyridoxal phosphate in the proposed sequential reactions of fatty acid elongation (cf. Scheme II) cannot be defined at present. The most likely possibility appears to be in the condensation step of acetyl CoA with the fatty acyl CoA acceptor, thus avoiding the requirement of the classical thiolase for this condensation reaction (Green, 1954; Lynen & Ochoa, 1953). As mentioned earlier, thiolase has two drawbacks; the first is the extremely low equilibrium constant of the condensation reaction ($1{\cdot}6 \times 10^{-5}$ M) at pH 7·0 (Lynen & Decker, 1957), and the second is the greater tendency of thiolase to catalyze the condensation of two acetyl CoA molecules to form acetoacetyl CoA rather than the condensation of acetyl CoA with a higher acyl CoA (Stansly & Beinert, 1953; Cornforth, 1959). The latter tendency appears to be the reason why Stansly & Beinert were unable to accumulate fatty acyl CoA's higher than butyryl CoA. The possible involvement of pyridoxal phosphate in this reaction may overcome these drawbacks, first by introducing specificity to the reaction in favor of condensation of acetyl CoA on an intermediate chain acyl CoA: and second by the formation of a complex between pyridoxal or pyridoxamine moiety and acetyl CoA resulting in the formation of a Schiff base (cf. Fig. 4) similar to the general pyridoxal coenzyme models proposed

by Snell (1958) and his colleagues (Westheimer, 1959; Braunstein, 1960). Such a complex may result in activating the methyl group of acetyl CoA by imposing an electronegative charge on the carbon atom and thus favoring its condensation with the relatively positively charged carbonyl group of acyl CoA resulting in the elongation of the carbon chain as shown in Scheme III.



SCHEME III. A hypothetical mechanism of action of pyridoxal phosphate in fatty acid elongation. R′ represents the rest of the pyridoxamine phosphate solution.

Thus, the role of pyridoxal phosphate in this reaction may be another means for the specific activation of the methyl group of acetyl CoA, other than the formation of malonyl CoA. The ultimate result would be in changing the equilibrium of the reaction in favor of carbon–carbon chain formation rather than cleavage.

Another alternative for the action of pyridoxal phosphate in fatty acid synthesis may be in the reduction of the α-β-unsaturated fatty acyl CoA derivatives by TPNH. This possibility appears very unlikely at present and must await further experimentation.

THE NON-MITOCHONDRIAL SYSTEM FOR FATTY ACID SYNTHESIS

A decade ago Gurin and his co-workers (Brady & Gurin, 1952; Dituri & Gurin, 1953; Van Baalen & Gurin, 1953) reported the synthesis of long-chain fatty acids from acetate, catalyzed by soluble extracts prepared from pigeon liver. Treatment of such extracts with charcoal resulted in a demonstrable requirement for ATP, DPN^+ and CoA. The addition of citrate to this system stimulated the process as did Mg^{2+}; acetyl CoA, however, was less efficient as a precursor of fatty acid synthesis than acetate. This system incorporated labeled acetate predominantly into fatty acids rather than into glycerides.

The observations of Gurin and his collaborators formed the basis for our studies on the mechanism of fatty acid synthesis (Wakil *et al.*, 1957; Porter *et al.*, 1957; Porter & Tietz, 1957; Gibson *et al.*, 1958a, b; Wakil *et al.*, 1958; Wakil, 1958; Wakil & Ganguly, 1959). This enzyme system may be prepared from chicken liver (Tietz, 1957), rat liver, and rat kidney, as well as from pigeon liver. When any of these tissues were extracted in a Potter–Elvehjem homogenizer with 0·1 M phosphate, 0·25 M sucrose or 0·88 M sucrose and the extract fractionated into mitochondria, microsomes, and soluble fractions, the soluble fraction invariably contained all the enzymes required for the conversion of acetate or acetyl CoA to fatty acids (cf. Table 4).

TABLE 4—INCORPORATION OF LABELED ACETATE WITH FATTY ACID BY PIGEON LIVER FRACTIONS

Fraction tested	mμmole of ^{14}C-acetate incorporated/hr per mg protein
Mitochondria	2·0
Microsomes	0·8
Clear supernatant	18·0
Mitochondria + microsomes	1·2
Mitochondria + clear supernatant	5·0
Microsomes + clear supernatant	8·0
Pellets	42·0

Each reaction mixture contains 5·0 μmole of acetate-1-^{14}C (300,000 c.p.m. total activity), 50 μmole of potassium phosphate buffer, pH 6·5, 4 μmole of ATP, 0·3 μmole of Mn^{2+}, 0·1 μmole of TPN^{+}, 8 μmole of isocitrate, 0·04 μmole of CoA, 6·0 μmole of cysteine and 10 μmole of $KHCO_3$. Final volume was 0·5 ml and the reaction was started by the addition of 2·5 mg of protein of each fraction where indicated. The reaction mixture was incubated at 38° for 2 hr. The pellets were obtained by further centrifuging the clear supernatant solution for 2 to 3 hr at 140,000 × g.

The 100,000 × g supernatant fraction shows higher activity per mg of protein than the whole homogenate minus cell debris. This is probably due to the removal of the inactive particles (mitochondria and microsomes) from the mixture. The results in Table 4 also show that addition of mitochondria or microsomes decreased the activity of the supernatant fluid.

A similar distribution of fatty acid synthesizing system was found by Brady *et al.* (1956) in pigeon liver, by Langdon (1957) in rat liver, and by Popják & Tietz (1955; Tietz & Popják, 1955) in lactating mammary gland. Therefore, we would like to designate this pathway as the "non-mitochondrial system" in order to differentiate it from the mitochondrial system discussed previously. Klein (1957) studied the fatty acid synthesizing system from yeast (*S. cervisiae*) and reported that the conversion of acetate to long-chain fatty acids required both the soluble fraction plus what he called "small particle fraction" (sedimented between 25,000

and 60,000 × g), free from the cytochromes and cytochrome oxidase. These results support our conclusion that the fatty acid synthesizing system is a non-mitochondrial system in origin.

The fatty acid synthesizing system from pigeon liver, rat livers, or rat kidney can be separated from the 100,000 × g supernatant fluid by centrifugation of this extract at 140,000 × g for 2–4 hr (Gibson *et al.*, 1958b). The precipitated particles (red-colored pellets) contained all the enzymes required for the conversion of acetate to fatty acids (cf. Table 4), while the supernatant fluid was devoid of activity. The structural relationships of the various enzymes of the fatty acid synthesizing sequence in these pellets are not as yet understood and must await further experimentation.

Extracts of pigeon liver were fractionated with ammonium sulfate into two fractions (Wakil *et al.*, 1957), the first (designated as R_1) is precipitated between 0 and 25% saturation of ammonium sulfate and the second fraction (designated as R_2) is precipitated between 25 to 40 per cent saturation.

R_1 and R_2 were both further purified by adsorption on calcium phosphate gel and subsequent elution with phosphate buffer (Wakil *et al.*, 1957; Gibson *et al.*, 1958b), followed by chromatography on a cellulose column (DEAE) according to the general procedure of Sober & Peterson (1958). The purified fractions thus obtained are referred to as R_{1gc} (derived from R_1 fraction) and R_{2gc} (derived from R_2). Further purification of the R_{2gc} fraction was effected by chromatography on calcium hydroxy apatite (Tiselius *et al.*, 1959) and the active enzyme preparation thus obtained designated as R_{2a}. The overall purification of the R_{2a} fraction amounts to about 3000-fold based on the original 100,000 × g supernatant extract (cf. Table 5).

TABLE 5—PURIFICATION OF THE R_{2a} ENZYME FRACTION

	Fraction	Specific activity (mμmole TPNH oxidized per min/mg protein)	Recovery of units (per cent)
100,000 × g soluble extracts	R_1	0·1	100*
Ammonium sulfate fraction 25–40%	R_2	1·0	80
Calcium phosphate gel eluate	R_{2g}	20·0	65
Fractionation on DEAE column	R_{2gc}	60·0	30
Fractionation on calcium hydroxy apatite	R_{2a}	300	25

* Arbitrarily taken as equal to 100

Products of the reactions

Earlier studies with whole animals (Schoenheimer & Rittenberg, 1937; de Stetten & Schoenheimer, 1940; Klein, 1943; Rittenberg & Bloch, 1944, 1945; Stevens & Chaikoff, 1951; Zabin, 1951; Anker, 1952; Dauben *et al.*, 1953;

Conigliso & Cate, 1958), tissue slices (Brady & Gurin, 1950) and micro-organisms (Smedley-Maclean & Hoffer, 1926; Sonderhoff & Thomas, 1937) support the concept that fatty acids are synthesized by successive head-to-tail condensation of two carbon units. Brady & Gurin (1952) separated the long-chain fatty acids ($> C_{10}$) that were synthesized by soluble preparations of pigeon liver, and on decarboxylation of these fatty acids (synthesized from acetate-1-^{14}C) the radioactivity in the carboxyl carbon atom (c.p.m. per C) of the fatty acid was found to be only slightly above the specific activity of the fatty acid (c.p.m. per C) which result would be expected from successive condensations of acetate units. Similar results were obtained by Popják & Tietz (1955); Tietz & Popják (1955) in mammary gland homogenates, by Klein (1957) in soluble extracts from yeast and by Stumpf & Barber (1956) utilizing extracts of avocado mesocarps.

The fatty acids synthesized by the highly purified enzymes were isolated and identified by a variety of techniques (Reid & Ledener, 1951; Kaufmann & Nitsch, 1954). The major components were palmitic acid ($>$ 80 per cent) with stearic and myristic acids constituting the remainder; short-chain acids (C_4 to C_{10}) did not accumulate under the reaction conditions employed (Porter & Tietz, 1957).

The palmitic acid synthesized from 1-^{14}C-acetate was isolated by dilution with unlabeled palmitic acid and recrystallized from various solvents to a constant specific activity. The resulting acid was decarboxylated by the Schmidt reaction as described by Phares (1951) and the liberated CO_2 was found to contain twice the average specific radioactivity of the other carbon atoms of the palmitic acid indicating a *de novo* synthesis of palmitic acid from acetate.

Acetyl CoA carboxylase

The discovery that bicarbonate was an absolute requirement for fatty acid synthesis can be regarded as the breaking point from the then prevailing concept that fatty acid synthesis proceeded via the reversal of β-oxidation. The significance of bicarbonate in fatty acid synthesis was first uncovered by Gibson *et al.* (1958a, b) who assigned a catalytic role for this metabolite in the conversion of acetyl CoA to palmitate. Earlier reports by Klein (1957) on the requirement of CO_2 in synthesis of fatty acids by cell-free preparations from yeast, and by Brady & Gurin (1950) and his co-workers on the stimulating effects of bicarbonate buffers on synthesis of fatty acids in liver slices, were not appreciated at the time and were attributed to non-specific effect. Gibson *et al.* (1958a, b) reported an absolute requirement for CO_2 in fatty acid synthesis by not only the highly purified enzyme preparations from pigeon liver but also by crude preparations of the enzyme from rat liver and rat kidney. A similar requirement for bicarbonate was reported by Stumpf and co-workers (Squires *et al.*, 1958) in their studies on fatty acid synthesis in extracts from avocado fruit. This common requirement for fatty acid synthesis strongly supports the thesis of the universality of this fatty acid synthesizing system in living cells. $H^{14}CO_3^-$ did not incorporate into the fatty acids during active synthesis from unlabeled acetyl CoA as shown in Table 6. This is not surprising, since it had been shown earlier by degradation studies that acetate or

acetyl CoA was the sole source of the carbon chain of the fatty acid synthesized by this system and for this reason a "catalytic" role for HCO_3^- has been proposed (Gibson *et al.*, 1958b).

TABLE 6—FAILURE OF $H^{14}CO_3^-$ TO BECOME INCORPORATED INTO THE SYNTHESIZED FATTY ACIDS

Expt. no.	Complete system plus	TPNH (-A 340mμ)	AcCOA incorp. into F.A. (c.p.m.)	$H^{14}CO_3^-$ incorp. into F.A. (c.p.m.)
1	Acetyl-l-^{14}C CoA and unlabeled HCO_3^-	0·460	8430	—
	Unlabeled acetyl CoA and $H^{14}CO_3^-$ (6.10^5 counts/min)	0·420	—	50
2	Acetyl-l-^{14}C CoA and unlabeled HCO_3^-	0·375	6800	—
	Unlabeled acetyl CoA and $H^{14}CO_3^-$ (2.10^6 counts/min)	0·370	—	0·00

Each cuvette contained the following components of the complete system: 25 μmole of potassium phosphate, pH 6·5, 1 μmole ATP, 0·3 μmole of $MnCl_2$, and 0·05 μmole of TPNH. As indicated the following reagents were also added: 50 mμmole of acetyl-l-^{14}C CoA (37,000 counts/min) or 45 mμmole of unlabeled acetyl CoA, 4 μmole of either unlabeled HCO_3^- or $H^{14}CO_3^-$. Total volume was 0·5 ml. The reaction was started by the addition of 0·6 mg of R_{1g} and 0·5 mg R_{2g}. The change in optical density at 340 mμ was followed for 5 min at 30° in the Beckman DUR and the amount of fatty acid synthesis was determined as usual at the end of this time.

The first intermediate in the synthesis of long-chain fatty acids from acetyl CoA was isolated by Wakil (1958). Incubation of acetyl CoA with the enzyme fraction R_{1gc} in the presence of HCO_3^-, Mn^{2+}, and ATP led to the formation of an intermediate which was isolated from the reaction mixture and which could be converted to palmitate in the presence of the second enzyme fraction R_{2gc} and TPNH thus splitting the reaction sequence into two parts. This intermediate incorporated both ^{14}C-acetyl CoA and $H^{14}CO_3^-$ in a ratio of approximately 1 : 1. After hydrolysis with alkali, the saponifiable fraction was extracted with diethyl ether and was shown to contain all of the radioactivity. This compound was identified as malonate (Wakil, 1958) by its R_f in two different chromatographic systems, melting point, and melting point of the ρ-nitrobenzyl ester. Formica & Brady (1959) were also able to demonstrate the similar formation of malonyl CoA from acetyl CoA catalyzed by crude extracts of pigeon liver and pig heart.

The formation of the malonyl derivative by the purified enzyme preparation R_{1gc} is dependent on the presence of Mn^{2+}, ATP and HCO_3^- (cf. Table 7).

Thus it appears that ATP activates the HCO_3^- (or any one of its equilibrium species, CO_2 and H_2CO_3) which is then condensed with the methyl group of

acetyl CoA to form malonyl CoA. For this reason the name *acetyl CoA carboxylase* (referred to previously as R_{1gc}) has been suggested for this enzyme.

TABLE 7—COMPONENTS OF ACETYL CoA CARBOXYLASE

	Malonyl CoA formed (mμmole)
Complete system	12·0
No AcCoA	0·0
No ATP	0·0
No HCO_3^-	0·1
No Mn^{2+}	0·1
No enzyme	0·0

The complete system contained 20 μmole acetyl CoA, 1·0 μmole ATP, 0·3 μmole $MnCl_2$, 4 μmole $KHCO_3$, 30 μmole phosphate buffer, pH 6·5, and 0·100 mg of acetyl CoA carboxylase in a total volume of 0·4 ml. The reaction mixture was incubated for 10 min at 38° and the reaction was stopped by heat denaturation of the enzyme. Malonyl CoA was assayed by its oxidation of TPNH in the presence of the R_{2gc} enzyme fraction.

The carboxylation reaction was absolutely dependent upon ATP for the activation of CO_2 prior to its condensation with acetyl CoA. The stoichiometry of the reaction showed that for each mole of malonyl CoA formed there was one mole of ATP utilized and one mole of each ADP and inorganic phosphate formed as shown in the following equation:

$$CO_2 + CH_3\overset{O}{\overset{\|}{C}}SCoA + ATP \xrightarrow[(\text{bound biotin})+Mn^{2+}]{\text{Acetyl CoA carboxylase}} CH_2\begin{matrix}COOH\\COSCoA\end{matrix} + ADP + P_i$$

Earlier reports on the stoichiometry of the overall conversion of acetyl CoA to palmitate based on experiments performed with relatively crude fractions of R_{1g} and R_{2g} indicated that two moles of ATP were utilized for every mole of acetyl CoA converted to fatty acids (Wakil *et al.*, 1959). When the highly purified acetyl CoA carboxylase was used in the malonyl CoA formation, only one mole of ATP was consumed for every mole of acetyl CoA carboxylated to malonyl CoA. Similar results were reported by Lynen (1959) and his colleagues (Lynen *et al.*, 1960) using purified enzyme preparations from yeast cells.

THE ROLE OF BIOTIN IN FATTY ACID SYNTHESIS (BIOTIN IN ACETYL CoA CARBOXYLASE)

Available information about the role of biotin in metabolic reactions points to a close relationship between this vitamin and the metabolism of carbon dioxide in several carboxylation–decarboxylation type reactions (for comprehensive review see György, 1954). A relationship has also been reported between biotin and fatty acid metabolism. Unsaturated fatty acids such as oleic, linoleic, etc., can promote growth of many micro-organisms in the absence of biotin (Broquist & Snell, 1951).

Saturated fatty acids, although inactive alone, have a sparing effect on the unsaturated fatty acids. Until recently (Wakil *et al.*, 1958; Wakil & Gibson, 1960), this relationship between biotin and fatty acid metabolism was very obscure and could not be readily related to the more generalized effect of biotin on the metabolism of carbon dioxide. Thus the absolute requirement for bicarbonate in the overall pathway of fatty acid synthesis by a purified enzyme system suggested a possible role for biotin. Assays of enzyme fractions for biotin content were performed by the procedure of Wright & Skeggs (1944; Wright *et al.*, 1952) and a significant concentration of this vitamin in the acetyl CoA carboxylase was found (Wakil *et al.*, 1958; Gibson *et al.*, 1958b; Wakil & Gibson, 1960). Biotin concentrated with the active protein of this fraction throughout the steps of purification (Wakil *et al.*, 1958) and the ratio enzymatic activity to biotin content remained essentially the same throughout the purification. The final concentration of biotin in the most purified preparation (after purification by ion exchange chromatography) amounts to about 2 or 3 mole of biotin per 10^6 g of protein or 1 mole of biotin per 300,000 to 500,000 g of protein.

Biotin is tightly bound to the protein and can be released only by tryptic digestion or acid hydrolysis (Wakil & Gibson, 1960). Preliminary results indicate that the product after digestion with trypsin and chymotrypsin appears to be both free and conjugated derivatives of biotin, possibly biocytin (Wakil & Gibson, 1960) as shown by microbiologic assay (Wright & Skeggs, 1944) and paper chromatography.

Wakil *et al.* (1958); Wakil & Gibson (1960) have presented evidence to show that the protein-bound biotin participates in the overall synthesis of long-chain fatty acids from acetyl CoA. The conversion of acetyl CoA to palmitate was inhibited by avidin (György, 1954), an egg-white protein which specifically binds biotin (cf. Fig. 5) and this inhibition could be relieved by the addition of free d-biotin as shown in Fig. 5. This technique, which was first used successfully by Wakil *et al.* (1958) has proved to be a useful technique in studying biotin enzymes (Wakil & Gibson, 1960; Lynen *et al.*, 1959; Kaziro *et al.*, 1960; Swick & Wood, 1960; Halenz & Lane, 1960; Lane *et al.*, 1960).

Biotin-deficient animals (rats and chicks) were prepared (Wakil *et al.*, unpublished observation) and their livers were used to prepare the fatty acid synthesizing system. The results show that the level of acetyl CoA carboxylase in the livers of these animals is lower than the amount of carboxylase in the normal animals but it is by no means absent. Purification of the enzyme from deficient animals was carried out, and at the highest purity level, the biotin content of such enzyme preparations appeared to be comparable to the preparations from normal animals. This observation indicates that biotin deficiency, however severe it may be, results in the decrease of the total level of the acetyl CoA carboxylase but it does not completely eliminate this vital enzyme from the liver.

Lynen (1959) and his collaborators (Lynen *et al.*, 1959) have recently isolated another biotin-containing enzyme which carboxylates β,β-dimethyl acrylyl CoA to form β-methyl glutaryl CoA. They presented evidence to show that biotin is an

integral part of this enzyme and that CO_2-biotin–enzyme is an intermediate in this reaction. The present evidence supports the following concerted mechanism of action of the biotin–enzymes (Kaziro *et al.*, 1960; Lane *et al.*, 1960):

$$CO_2 + ATP + \text{biotin–enzyme} \longrightarrow CO_2 \sim \text{biotin–enzyme} + P_i + ADP$$

$$CO_2 \sim \text{biotin–enzyme} + \text{acceptor} \longrightarrow HOOC\text{–acceptor} + \text{biotin–enzyme}$$

The acceptor may be acetyl CoA, propionyl CoA, β,β-dimethylacrylyl CoA, etc.

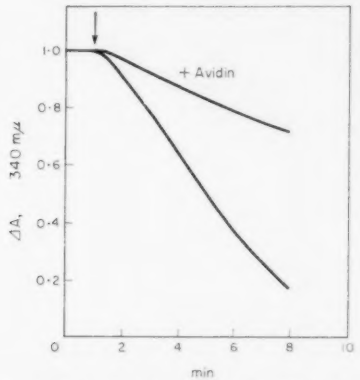


FIG. 5. Avidin inhibition of the oxidation of TPNH associated with fatty acid synthesis: purified avidin (46 μg) was added to one of two identical systems each of which contained in a total volume of 0·50 ml the following reagents—potassium phosphate buffer (pH 6·5), 50 μmole, $MnCl_2$, 0·3 μmole, ATP, 1·0 μmole, acetyl CoA, 0·21 μmole, $KHCO_3$, 5·0 μmole, TPNH, 0·17 μmole and R_{2g}, 1·5 mg. The reaction was initiated in both systems by the addition of 0·26 mg R_{1g} (indicated by arrow). The optical density at 340 mμ (1·0 cm cell) was followed continuously in the Beckman DUR spectrophotometer) at 38°).

Conversion of malonyl CoA to palmityl CoA

The conversion of malonyl CoA to palmitate was shown (Wakil, 1958; Wakil & Ganguly, 1959) to be catalyzed by the second enzyme fraction (R_{2gc}) in the presence of TPNH. Similar results were obtained by Brady (1958, 1960; Brady *et al.*, 1960) in crude pigeon liver preparations, by Lynen (1959) and his colleagues in yeast extracts (Lynen *et al.*, in press), by Stumpf and co-workers in avocado mesocarp (Squires *et al.*, 1958), and by Vagelos and his colleagues in adipose tissue (Horning *et al.*, 1960). Furthermore, Ganguly (1960) was able to demonstrate the presence and the relative distribution of this system in various tissues.

Fatty acid synthesis from malonyl CoA could be followed either spectrophotometrically by measuring the oxidation of TPNH or isotopically by measuring the incorporation of ^{14}C-labeled malonyl CoA into palmitate. The requirement for acetyl CoA in the conversion of malonyl CoA to palmitate is absolute as shown by Fig. 6. Furthermore, a significant amount of ^{14}C-acetyl CoA was incorporated into palmitate when unlabeled malonyl CoA was used, shown in Table 8.

The tendency of the reaction to proceed toward palmitate synthesis after incubation of malonyl CoA with enzyme in absence of acetyl CoA (as shown in Fig. 6) may result from the presence of a contaminating enzyme in the R_{2a} preparation which decarboxylates malonyl CoA to acetyl CoA plus CO_2. Indeed such an

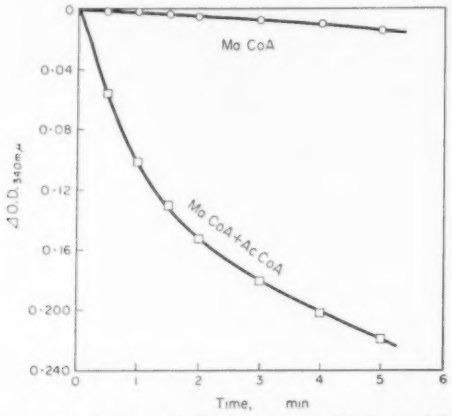


FIG. 6. The requirement for acetyl CoA in the oxidation of TPNH by malonyl CoA. Each cuvette contained 30 μmole of phosphate buffer, pH 6·5, 0·015 μmole acetyl CoA where indicated, 0·016 μmole malonyl CoA, 0·05 μmole TPNH and water to a final volume of 0·4 ml. The reaction started with the addition of 20 μg of R_{2a} fraction.

enzyme could be demonstrated in the cruder fractions of R_{2gc} by the formation of citrate from malonyl CoA in the presence of oxaloacetate and Ochoa's condensing enzyme (Ochoa *et al.*, 1951).

The data in Table 8 also show the stoichiometric relationship (based on 1 mole of palmityl CoA formed) between acetyl CoA, malonyl CoA and TPNH in the synthetic process. The results show that for each mole of palmityl CoA synthesized, 1 mole of acetyl CoA, 7 mole of malonyl CoA and 14 mole of TPNH are consumed and 7 mole each of CO_2, CoA and water were formed. The overall reaction can be presented as follows:

$$OOCCH_2\overset{O}{\overset{\|}{C}}SCoA + 14\,TPNH + 14H^+ \longrightarrow CH_3(CH_2)_{14}\overset{O}{\overset{\|}{C}}SCoA$$
$$+ 14\,TPN^+ + 7CO_2 + 7CoASH + 7H_2O$$

In short, one "C_2-unit" of palmitate is derived from acetyl CoA and the remaining 14 carbon atoms are derived from malonyl CoA.

Acetyl CoA contributes carbons 15 and 16 of palmitate as shown by degradation studies of the carboxyl group of palmitic acid derived from 1-^{14}C-acetyl CoA and 1-^{14}C-malonyl CoA respectively. The carboxyl group of palmitic acid derived from 1-^{14}C-acetyl CoA had no radioactivity while the carboxyl group of palmitic acid synthesized from malonyl CoA ($HOOCCH_2{}^{14}COSCoA$) had the same specific activity as that of the labeled carbonyl group of malonyl CoA.

Acetyl CoA can be substituted for by propionyl CoA and to a lesser degree by longer-chain fatty acyl CoA's, such as hexanol or octanoyl CoA. 1-^{14}C-butyryl CoA is incorporated into palmitate to low extent. This appears to be contrary to

TABLE 8—STOICHIOMETRY OF THE PALMITYL CoA FORMATION FROM MALONYL CoA PLUS ACETYL CoA

AcCoA (mμmole)	MaCoA (mμmole)	TPNH (mμmole)	Palmitate (mμmole)	CO_2 (mμmole)	CoASH (mμmole)
	−8·2	−16·7	+1·1		+7·9
	−6·0	−12·3		+5·8	
−2·2		−25·8	1·9		

Three separate experiments were used for the determination of the stoichiometry of the reaction. In the first experiment the reaction mixture contained 8·5 mμmole ^{14}C-malonyl CoA ($HOOCCH_2$ ^{14}COCoA, 20,000 c.p.m.), 50 μmole TPNH, 13 mμmole acetyl CoA, 30 μmole phosphate buffer, pH 6·5, 0·120 mg of R_{2gc} and water to a final volume of 0·4 ml. The oxidation of TPNH was followed spectrophotometrically (cf. Fig. 10). At the end of the reaction (5 min, temp. 38°), aliquots were withdrawn for (1) Co ASH analysis, (2) palmitate analysis by ^{14}C-incorporation and (3) malonyl CoA as determined by the amount of ^{14}C left in the reaction mixture after extraction of ^{14}C-palmitate.

In the second experiment, the reaction mixture contained exactly the same reagents as in the first experiment except that 6·0 mμmole carboxyl-labeled malonyl CoA ($HOOCCH_2$ ^{14}COCoA, 13,000 c.p.m.) were used. The reaction was followed by TPNH oxidation. The $CO_2{}^{14}$ was trapped in NaOH and the radioactivity was determined as $BaCO_3{}^{14}$. Palmitate did not incorporate any ^{14}C.

In the third experiment, the reaction mixture was the same as in the first experiment except that 13 mμmole 1-^{14}C-acetyl CoA (32,000 c.p.m.) and 30 mμmole of unlabeled malonyl CoA were used. The remaining acetyl CoA was determined by both radioactivity and by the citrate-condensing enzyme.

our earlier reports, based on experiments utilizing cruder enzyme preparations, that acetyl CoA can be substituted for by butyryl CoA or octanoyl CoA (Wakil & Ganguly, 1959). The pathway of incorporation of butyryl CoA or octanoyl CoA into palmitate in these experiments is not clear as yet; nevertheless with highly purified subfractions of R_2 only acetyl CoA or propionyl CoA can be readily incorporated into long-chain fatty acids. When propionyl CoA was used instead of acetyl CoA, the product of the synthesis (70 per cent) was an odd chain fatty acid with 17 carbon atoms, as shown by analysis of the products in gas chromatography. This observation, therefore, may explain the occurrence of odd chain fatty acids in animal tissues. This appears to be dependent on the availability of propionyl CoA to the cell rather than on the presence of another enzyme system for the synthesis of odd chain fatty acids. Horning *et al.* (1960) arrived independently at the same conclusion.

When butyryl CoA was used instead of acetyl CoA the main product of the synthesis appeared to be stearic acid (85 per cent) in contrast to palmitic acid as the main product of synthesis when acetyl CoA was used. In all these cases, it appears

that ultimately 7 malonyl CoA molecules were added on to each fatty acyl CoA to yield the corresponding long-chain fatty acids. The significance of this observation cannot be evaluated fully at this time; one possibility may be due to the inherent specificity of the enzyme preparation which catalyzes the addition of 7 malonyl CoA molecules regardless of the length of the short-chain fatty acyl CoA's.

When the rate of the palmitate formation, as measured by the rate of oxidation of TPNH, was plotted against the concentration of acetyl CoA, propionyl CoA, or butyryl CoA, the usual hyperbolic plot was obtained (cf. Fig. 7). The same data

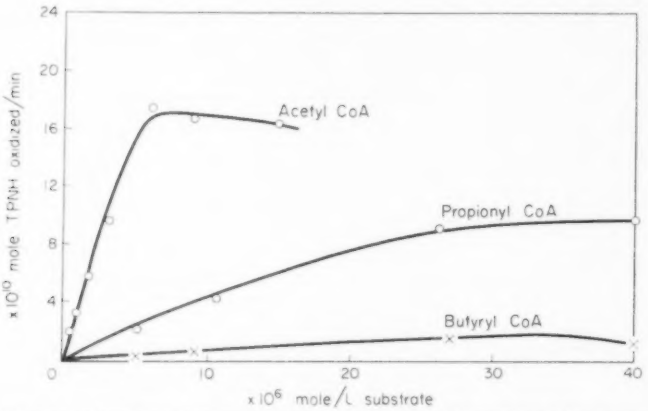


FIG. 7. The relationships between the rate of fatty acid synthesis (as measured by the rate of TPNH oxidation) and acetyl CoA, propionyl CoA or butyryl CoA concentrations. Each reaction mixture contained 30 μmole of phosphate buffer (pH 6·5), 0·016 μmole malonyl CoA, 0·05 μmole TPNH, the indicated amounts of acetyl CoA, propionyl CoA, or butyryl CoA and water to a final volume of 0·4 ml. The reaction started by the addition of 0·1 mg of R_{ggc}. Temperature was 38°. The rate of the reaction was linear for the first 3 min.

could be plotted by the method of Lineweaver & Burk (Fig. 8) yielding two straight lines which intercept the ordinate as a single point.

From such plots the Michaelis–Menten constants for acetyl CoA, propionyl CoA and butyryl CoA were determined and found to be $2{\cdot}3 \times 10^{-6}$M, $5{\cdot}7 \times 10^{-5}$M and $3{\cdot}9 \times 10^{-4}$M respectively. These values indicate that the affinity of the enzyme for acetyl CoA is about 25 times that of butyryl CoA.

The requirement for acetyl CoA and long-chain fatty acyl CoA in the synthesis of long-chain fatty acid from malonyl CoA has been confirmed by many laboratories (Lynen *et al.*, in press; Brady *et al.*, 1960; Brady, 1960; Horning *et al.*, 1960; Long & Porter, 1959; Gibson & Hubbard, 1960). Lynen and his associates presented data to show that the synthesis of long-chain fatty acids from malonyl CoA in the yeast preparations is directly proportional to the chain length of the fatty acyl CoA's added. For instance, they reported that butyryl CoA is a better "primer" for fatty acid synthesis than acetyl CoA and so on. Horning *et al.* (1960)

have reported that ^{14}C-labeled propionyl CoA, isobutyryl CoA, isocaproyl CoA, isovaleryl CoA and α-methylbutyryl CoA are incorporated into the long-chain fatty acids by extracts of epididymal adipose tissue. According to these authors the extent of fatty acid synthesis in the presence of the different acyl CoA compounds was essentially the same.

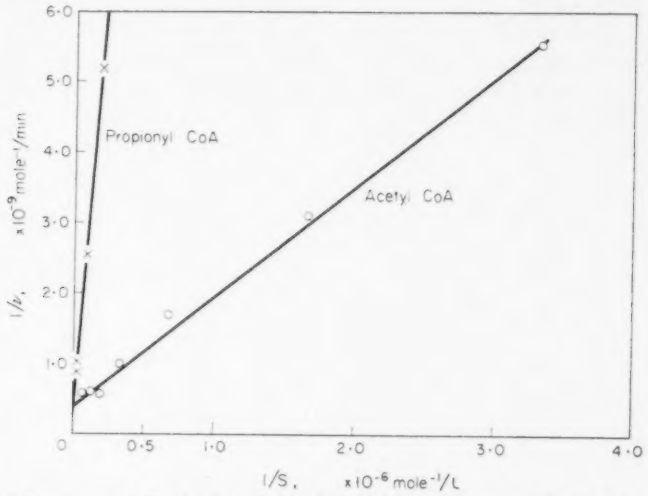


FIG. 8. Lineweaver & Burk plot of the data in Fig. 7 for acetyl CoA and propionyl CoA.

With the highly purified pigeon liver enzyme preparations this does not appear to be the case as shown by the *Km* values (cf. Figs. 7 and 8). These fatty acyl CoA's do indeed incorporate in significant amounts into the long-chain fatty acids but to a much lesser extent than acetyl CoA and propionyl CoA. The degree of incorporation of the short-chain fatty acyl CoA's appears to be inversely proportional to their chain length. Whether this behavior is peculiar to the pigeon liver enzyme or indicates the existence of more than one pathway cannot be determined as yet.

Condensation of acetyl CoA and malonyl CoA

In addition to the enzyme preparation are the substrates required for fatty acid synthesis, malonyl CoA, acetyl CoA and TPNH. The inability to demonstrate TPNH oxidation in the absence of either acyl CoA's suggested that condensation of acetyl CoA and malonyl CoA occurs prior to any reductive transformation.

Earlier attempts to follow and study such a condensation were unsuccessful because of the presence of various contaminating enzymes in the preparation of the fatty acid synthesizing system. Malonyl CoA decarboxylase (Hogeboom *et al.*, 1948; Hayashi, 1955), an enzyme which decarboxylates malonyl CoA to acetyl CoA and CO_2, is difficult to remove or separate completely. The best preparation obtained by us reduced this interfering enzyme to a level of 10 per cent of that of

the fatty acid synthesizing enzyme. With the aid of such preparations Wakil & Bressler (unpublished observations) were able to demonstrate the condensation of acetyl CoA and malonyl CoA with a concomitant release of CO_2. The reaction was followed conveniently by measuring $^{14}CO_2$ released from carboxyl-labeled malonyl CoA ($HOO^{14}CCH_2COSCoA$). Tenfold stimulation of CO_2 formation could be achieved by addition of acetyl CoA to reaction mixture containing malonyl CoA and enzyme R_{2a}. Under these conditions the liberation of $^{14}CO_2$ was proportional to both time and enzyme concentrations as shown in Figs. 9 and 10. The values reported in these figures were corrected for the acetyl CoA independent decarboxylation of malonyl CoA.

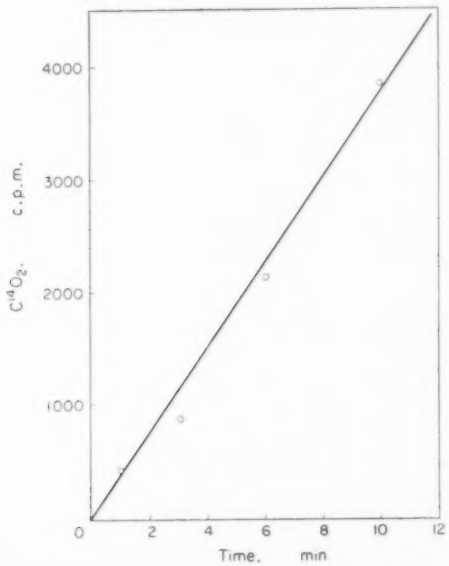


FIG. 9. The rate of acetyl CoA dependent decarboxylation of $HOO^{14}CCH_2COSCoA$. The reaction mixture contained 30 μmole potassium phosphate buffer (pH 6·5), 30 mμmole acetyl CoA, 100 mμmole $HOO^{14}CCH_2COSCoA$ (15,000 c.p.m.), 40 μg protein (R_{2a} enzyme) and water to a final volume of 0·5 ml. The reaction mixture was incubated at 30° for the time indicated and the $^{14}CO_2$ was trapped in hyamin and was measured in the Tricarb Scintillation Spectrometer. All values were corrected for the decarboxylation of malonyl CoA in the absence of acetyl CoA.

At optimal concentration of acyl CoA, propionyl CoA causes the release of 40 per cent as much $^{14}CO_2$ from malonyl CoA as does acetyl CoA while butyryl CoA causes the release of 4 per cent as much CO_2 as does acetyl CoA. These results confirmed our e.rlier observations on the incorporation and substitution of various chain-length fatty acyl CoA's (C_4 CoA, C_6 CoA, C_8 CoA, etc.) for acetyl CoA in the overall synthesis of long-chain fatty acids.

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The product of condensation was isolated from the reaction mixture by paper chromatography. A compound with an R_f of 0·73 in isobutyric $NH_3 \cdot H_2O$ (66 · 1 · 33) and 0·83 in 0·1 M sodium acetate : ethyl alcohol (1 : 1) was obtained

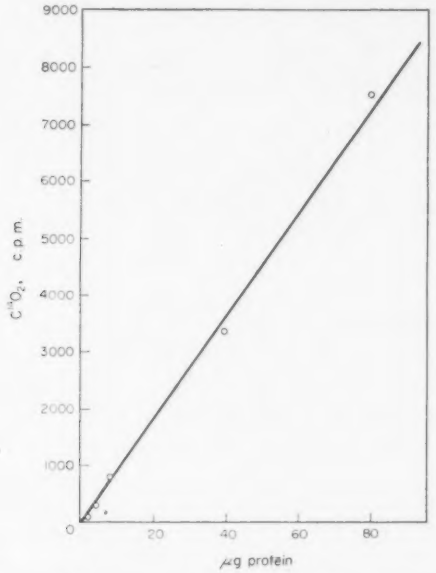


FIG. 10. The relationship between acetyl CoA dependent decarboxylation of $HOO^{14}CCH_2COSCoA$ and the amount of enzyme. The reaction was carried out exactly as described in Fig. 9 except for 10 min incubation at 30° and varying amounts of enzymes as indicated.

which was completely distinct from palmityl CoA, acetyl CoA, malonyl CoA and malonic acid. Hydrolysis of the compound yielded an acid which moved in the butanol : ammonia system of Reid & Ledener (1951) with an R_f of 0·41 (cf. Figs. 11 and 12), and was completely separable from acetoacetic, malonic, β-hydroxybutyric, acetic, butyric, hexanoic and palmitic acids. Both acetyl-1-^{14}C CoA and carbonyl-labeled malonyl CoA ($HOOCCH_2{}^{14}COSCoA$) were incorporated into this acid as shown in Figs. 11 and 12, while the carboxyl group of malonyl CoA ($HOO_2CCH_2COSCoA$) was not incorporated and was released as $^{14}CO_2$ during the condensation. The addition of TPNH to the reaction mixture did not result in accumulation of this compound, and, instead, palmitic acid was formed which, in agreement with the aforementioned stoichiometry, incorporated 1 mole of acetyl CoA and 7 mole of malonyl CoA. The absence of the presumed condensation product from reaction performed in the presence of TPNH suggested to us that this or a closely related compound was an intermediate in the conversion of malonyl CoA and acetyl CoA to palmitate. Failure to demonstrate the formation of this compound in the presence of TPNH may indicate that the initial condensation is rate determining for the overall synthetic sequence. Should the isolated

compound be unrelated to the conversion of malonyl CoA and acetyl CoA to palmitate, then a significant amount of it might have been expected to accumulate in the presence of TPNH. Attempts to isolate this compound in its active form and

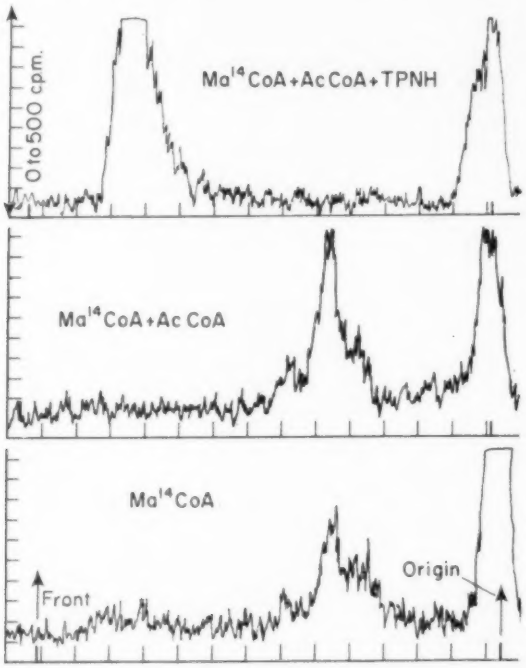


FIG. 11. Scanning of the paper chromatograms of the acid accumulated after the condensation of ^{14}C-malonyl CoA and acetyl CoA with the concomitant release of CO_2. Three separate tubes contained 30 μmole phosphate buffer (pH 6·5), 50 mμmole of $HOOCCH_2{}^{14}COSCoA$ (150,000 c.p.m.), 40 μg of R_{2a} enzyme, water to a final volume of 0·4 ml and where indicated the following were also added; 50 mμmole TPNH and 30 mμmole acetyl CoA. The reaction mixture was incubated for 10 min at 38°. At the end of the incubation the acids were extracted with diethylether after mild hydrolysis with alkali and were chromatographed in the butanol : ammonia system of Reid & Ledener (1951). The compound at the origin was malonic acid, the one nearest to the front was palmitic acid and the compound with R_f 0·4 was the unknown. No detectable compound was found when TPNH was added to the reaction mixture.

to convert it to palmitate by reduction with TPNH have not been successful. This may be a result of some chemical modification occurring during the isolation procedure. More likely, however, is turnover (enzymatic or otherwise) of an enzymatically reactive intermediate(s) in the absence of TPNH into the isolated compound plus "a cofactor". Preliminary observations indicate that a co-factor may be involved in the overall conversion of malonyl CoA and acetyl CoA to fatty acids.

Incorporation of tritiated acetyl CoA and malonyl CoA into palmitate

In 1937, Sonderhoff & Thomas demonstrated for the first time that deuterium-labeled acetic acid (CD_3COOH) could be incorporated into long-chain fatty acids

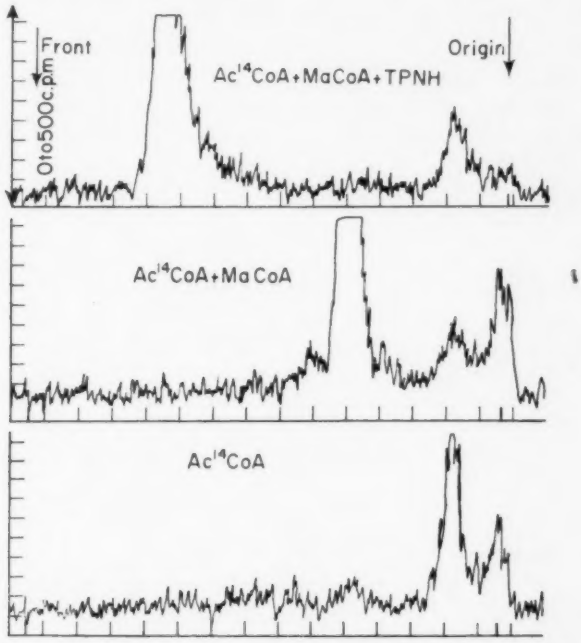


FIG. 12. Scanning of the paper chromatograms of the acid accumulated after the condensation of unlabeled malonyl CoA with ^{14}C-acetyl CoA. The same experiments were carried out as described in Fig. 11 except that unlabeled malonyl CoA and acetyl-l-^{14}C CoA (500,000 c.p.m.) were used.

by yeast cells. These results were confirmed by Rittenberg & Bloch (1944, 1945) and were extended to include experiments on whole animals (rats and mice) in which they showed that deuterium-labeled acetate incorporated in cholesterol as well as fatty acids. Recently we studied the incorporation of tritium-labeled acetyl CoA ($CT_3COSCoA$) and tritium-labeled malonyl CoA ($HOOCCT_2COSCoA$) into palmitate by the purified enzyme preparations derived from pigeon liver.

Tritiated acetyl CoA and malonyl CoA were prepared enzymatically with the aid of acetic thiokinase and acetyl CoA carboxylase respectively. When $CT_3COSCoA$ was added to a reaction mixture containing non-labeled malonyl CoA, TPNH and the R_{2a} enzyme, tritium-labeled fatty acids were isolated. The incorporation of tritium from acetyl CoA was absolutely dependent upon the presence of malonyl CoA. The amount of tritium incorporated into the palmitate corresponded to about 1 mole of acetyl CoA incorporated into 1 mole of palmitate (as measured by TPNH oxidation) or 3 atoms of tritium were incorporated per

molecule of palmitate as shown in Table 9. This is in complete agreement with the formulation given above for the synthesis of palmityl CoA from acetyl CoA and malonyl CoA. It would also indicate that the methyl group (CT_3) of acetyl CoA is incorporated into palmitate as a unit (presumably as carbon 16 of palmitate) with no loss of tritium atoms during this transformation.

TABLE 9—THE INCORPORATION OF TRITIUM-LABELED ACETYL CoA ($CT_3COSCoA$) AND MALONYL CoA ($HOOCCT_2COSCoA$) INTO PALMITATE

Exp. no.	Tritiated acyl CoA added	A	B	Ratio B/A
		Palmitic acid synthesized* mμmole	Tritium incorporated into palmitate mμatom	
I	$CT_3COSCoA$	3	9·45	3·15
II	$HOOCCT_2COSCoA$	4·7	32·2	6·9
III	$HOOCCT_2COSCoA$	1·4	6·9	5·0
IV	$HOOCCT_2COSCoA$	1·5	8·7	6·4

* The amount of palmitate synthesized was calculated from the total amount of TPNH oxidized in the reaction mixture.

In each sample the following reagents were added: 30 μmole potassium phosphate, pH 6·5, 50 mμmole TPNH, 20 to 40 μg protein (R_{2a}) and water to a final volume of 0·4 ml. In experiment I, 40 mμmole $CT_3COSCoA$ ($1·35 \times 10^6$ c.p.m./μmole) and 75 mμmole non-labeled malonyl CoA were added. In experiments II, III, and IV, 60 mμmole non-labeled acetyl CoA and 20 mμmole tritium-labeled malonyl CoA ($1·67 \times 10^5$ c.p.m./μmole) were added. The reaction mixture was incubated for 5 min at 38°.

When ($HOOCCT_2COSCoA$) was added to non-labeled acetyl CoA, TPNH and the R_{2a} enzyme, tritium-labeled palmitic acid was also isolated (cf. Table 9). The amount of tritium labeling in palmitic acid in three such experiments amounted to 5 to 6·8 μatoms of tritium per one μmole of palmitic acid synthesized. Since our stoichiometric formulation for palmitic acid synthesis indicated the consumption of 7 mole of malonyl CoA for each mole of palmityl CoA formed then it is reasonable to conclude that there was approximately one atom of tritium incorporated into palmitate per C_2 unit derived from malonyl CoA. In other words, of two possible tritium atoms that might incorporate into palmitate from $HOOCCT_2COSCoA$, only one tritium atom was found in palmitate while the other was presumably lost to the medium in the form of water (THO) during the course of the reaction.

These results did not lend support to our earlier suggestion that dicarboxylic acyl CoA's may be involved as intermediates in the conversion of acetyl CoA and malonyl CoA to palmitate. If the latter were the case, then we could not expect any incorporation of tritium atoms from malonyl CoA into palmitate. Therefore, a modification of the scheme of Wakil & Ganguly must be made in order to take these

observations into account. Such a modification must assume that the release of CO_2 from malonyl CoA takes place prior to the reduction of the carbonyl group, elimination of H_2O and the reduction of the carbon–carbon double bond. These assumptions are compatible with the aforementioned observation that CO_2 was liberated during the condensation of acetyl CoA and malonyl CoA.

The possible mechanisms of fatty acids' synthesis

In a preliminary communication (Wakil & Ganguly, 1959), we proposed a scheme for palmityl CoA synthesis from acetyl CoA and malonyl CoA which at the time appeared to explain our observations. This hypothesis proposed the formation of a C_5 intermediate (possibly acetomalonyl CoA) as the initial compound formed from the condensation of acetyl CoA and malonyl CoA. This C_5 compound was then thought to be successively reduced, dehydrated, reduced again and decarboxylated to yield butyryl CoA. Thus, in five successive steps, butyryl CoA was thought to be formed from acetyl CoA plus malonyl CoA. The butyryl CoA could then condense with malonyl CoA to yield a C_7 dicarboxylic acyl CoA which would undergo the same successive transformations to yield a C_6 CoA and the process would be repeated until finally palmityl CoA was formed. This hypothesis was based primarily on the ability of butyryl CoA and octanoyl CoA to be incorporated into palmitate and the inability of acetoacetyl CoA, β-hydroxyacyl CoA and crotonyl CoA to oxidize TPNH and be incorporated into palmitate.

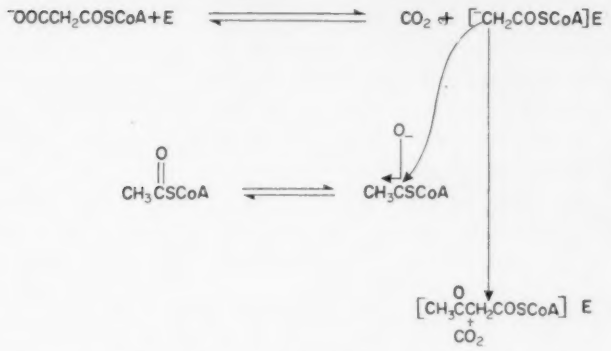
With the aid of enzyme preparations from yeast, Lynen *et al.* (in press) were able to confirm our observations on the inability of substituted acyl CoA's to oxidize TPNH and to incorporate into the fatty acids. These observations plus the fact that the fatty acid synthesizing system could be readily inhibited by sulfhydryl binding reagents (iodoacetamide, arsenite, ρ-chloromercuryribenzoate, etc.) and the tendency of malonyl CoA to bind onto the enzyme (an observation which we confirmed with our preparations) suggested to Lynen and his colleagues the possible formation of tightly bound acyl-S-enzyme as intermediates in the conversion of malonyl CoA and acetyl CoA to palmitate. Lynen's scheme assumed that malonyl-S-enzyme is first formed from malonyl CoA and enzyme-SH which is then condensed with an acyl CoA (acetyl CoA, butyryl CoA, hexanoyl CoA, etc.) and simultaneously decarboxylates to form a β-ketoacyl-S-enzyme. The latter derivative is then reduced, dehydrated and reduced again to form the saturated acyl-S-enzyme which would then undergo an ester interchange reaction with CoASH to yield acyl CoA and free HS-enzyme. This scheme assumed the presence of only one enzyme responsible for the entire sequence and required that the saturated acyl CoA's (C_4 CoA, C_6 CoA, etc.) were intermediates in the synthesis of palmityl CoA. Our recent observations on the mechanism of fatty acid synthesis appeared to be at variance with those reported by Lynen *et al.* (in press) especially as far as the relative degree of incorporation of the fatty acyl CoA's into palmitate were concerned. Whether these observations were peculiar to the pigeon liver enzyme preparation cannot be answered at the moment. They may be summarized

as follows; (1) Acetyl CoA, propionyl CoA and, to a lesser extent, butyryl CoA can be incorporated into long-chain fatty acids in the presence of malonyl CoA, TPNH and enzyme. (2) One mole of acyl CoA is incorporated into 1 mole of the fatty acid and constitutes the last 2, 3 or 4 carbon atoms respectively of the synthesized C_{16}, C_{17} or C_{18} fatty acid. (3) The ability of butyryl CoA and longer homologs of acyl CoA's to substitute for acetyl CoA is very small and it decreases sharply with increasing chain length. (4) Experiments (including trapping and dilution techniques) designed to isolate short-chain acyl CoA's (butyryl CoA, hexanoyl CoA, etc.) from the reaction mixture were not successful. (5) We have been unable to accumulate and isolate a dicarboxylic acyl CoA, and thus we were unable to confirm Steberl *et al.* (1960) who reported the isolation of a "C_5" and "C_7" dicarboxylic acyl CoA from the condensation of acetyl CoA and butyryl CoA with malonyl CoA respectively. (6) Substituted acyl CoA's (acetoacetyl CoA, β-hydroxybutyryl CoA, crotonyl CoA) do not oxidize TPNH in the presence of purified enzyme, nor do they incorporate into the fatty acid or dilute the amount of ^{14}C-malonyl CoA converted to palmitate. (7) For each mole of palmityl CoA synthesized, 1 mole of acetyl CoA, 7 mole of malonyl CoA and 14 mole of TPNH were consumed and 7 mole of CO_2, 7 mole of CoASH, 14 mole of TPN and 7 mole of water were formed. (8) Three tritium atoms from tritium-labeled acetyl CoA ($CT_3COSCoA$) were incorporated into 1 molecule of palmitate, indicating that the α-carbon of acetyl CoA constituted carbon 16 of palmitate. (9) One tritium atom of the tritium-labeled malonyl CoA $HOOCCT_2COSCoA$ was incorporated into palmitate per one "C_2-unit" derived from malonyl CoA while the other tritium atom was presumably lost as water during the reactions. (10) In the absence of TPNH, acetyl CoA and malonyl CoA condense to form a compound with a simultaneous release of CO_2. The addition of TPNH to the reaction mixture prevented completely the accumulation of this compound even under conditions where excess malonyl CoA and acetyl CoA were present. On hydrolysis of this compound an acid was isolated which could be demonstrated to have incorporated both acetyl CoA and malonyl CoA except for the carboxyl carbon of malonyl CoA which is released as CO_2 during the condensation. The nature of this compound is not known as yet. Further experimentations are necessary in order to ascertain the exact structure of this compound and its role in the overall synthesis of long-chain fatty acids.

The incorporation of 7 tritium atoms from triated malonyl CoA ($HOOCCT_2COSCoA$) into the palmitate molecule appeared to be of special significance in our understanding of the mechanism of fatty acid synthesis. Since CO_2 is released concomitant with the condensation of malonyl CoA onto acetyl CoA, it is conceivable that a negatively charged hypothetical intermediate such as this ($^-CH_2COSCoA$) may be formed on the enzyme surface as a result of the interaction of the enzyme with malonyl CoA. Such an intermediate is then coupled with the partially positively charged carbonyl group of acetyl CoA

$$CH_3\text{-}\overset{O^-}{\overset{|}{C}}{}^+\text{-}SCoA$$

to form the keto derivative according to the following formulation:



As stated above, very little is known about the nature of the β-keto derivatives. Preliminary observations indicate that it is probably not acetoacetyl CoA. Lynen has suggested that it is an acetoacetyl derivative of the enzyme (CH_3-C-CH_2-C-SE) which can be formed as a result of the condensation of acetyl CoA and malonyl-S-enzyme but cannot be formed by the interaction of acetoacetyl CoA and the enzyme. Other possibilities including the formation of a polyketo acyl CoA (Lynen, 1959) or an acyl derivative of a coenzyme other than coenzyme A cannot be ruled out at the present.

The loss of one of the tritium atoms of the α-carbon of malonyl CoA during the synthesis of palmitic acid as water represented the most direct evidence so far for the dehydration of the intermediate(s) during the sequential transformation of malonyl CoA to fatty acids. The elimination of water would undoubtedly yield an α-β-unsaturated acyl compound which is then reduced by TPNH to form the saturated derivative. It is too early as yet to say whether the reduction of ($>C=C<$) by TPNH takes place by direct transfer of electrons from the pyridinonucleotide or through the intermediate oxido–reduction of another coenzyme (such as flavins in flavoproteins). It is interesting to note in this regard that all the previously reported oxide–reduction reactions of DPN^+ and TPN^+ involve substrates with substituted carbons (e.g. CHOH, $CHNH_2$, etc.) and none that are —CH=CH—. On the other hand, there are numerous examples where flavoproteins are involved in the oxido–reduction of the carbon–carbon bond.

Further information is necessary in order to substantiate this mechanism. One of the attractive hypotheses is that proposed earlier by Lynen and later dropped in favor of the acyl-S-enzyme. This involves the formation of a polyketo polymer of C_2 units (polyketo palmityl CoA) which is then reduced completely to palmityl CoA. Such a compound might be formed by the condensation of 1 mole of acetyl CoA and 7 mole of malonyl CoA with the release of 7 mole of CO_2 and CoASH. The polyketo palmityl CoA formed could be reduced by TPNH to palmityl CoA. Although it is very difficult to conceive of the complete enzymic reduction of the polyketo acid occurring in a single step, it is conceivable that such

a reduction of the undoubtedly predominant enol form may take place with the simultaneous removal of water, provided that during the tautomeric shifts there were no loss of tritium from the methyl group of the polyketo acid. The relationship of the isolated compound to the proposed polyketo acid is not yet determined. It is conceivable, however, that the isolated compound is the inactive form of the polyketo acid that has lost its active co-factor.

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TRANSFER AND STORAGE OF LIGHT ENERGY IN PHOTOSYNTHESIS

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1. PRIMARY PHOTOCHEMICAL PROCESS IN CONDENSED SYSTEMS

In the study of gases, we are accustomed to divide the photochemical process into the primary act and the secondary "dark" reactions. Typically, the primary photochemical process may be dissociation of the light-absorbing molecule into atoms (or, more generally, free radicals) or it may be transformation into an unstable and (in principle) fluorescent form, which survives—in the case of strongly absorbing molecules—for 10^{-7} sec or less. After this, the molecule either loses all its excitation energy, or is converted into a "metastable" excited form (such as the triplet form—if the ground state and the fluorescent state are singlets), in which it retains a large part of its excitation energy for 10^{-5} to 10^{-3} sec, or even longer. The secondary dark reactions are the chemical reactions initiated either by the free atoms or radicals, or by the excited—short-lived or metastable—molecules.

In condensed phases, the mechanism of photochemical reactions is complicated by several phenomena, including *primary recombination* of atoms or radicals ("cage effect"): *complex formation* between the light-excited molecule and other reactants, and *energy transfer* between the light-excited and other nearby—identical or different—"resonating" molecules. In the limiting case of crystalline solids, *ionization* of the excited molecules also becomes possible, producing a "free" electron and a positive "hole" (which, too, may be more or less freely movable in the solid). This ionization can be produced by light quanta much smaller than those needed to ionize the same molecule in the gaseous state, because the "free" electron is not truly free, but stabilized by the crystal field. While it is not attached to an individual molecule in the lattice, it still belongs to the crystal as a whole.

These phenomena, except the last two, can occur, under appropriate conditions, also in gases, but they are particularly typical of the photochemistry of condensed systems, especially those which contain a high average concentration (or show a high local accumulation) of the light-absorbing molecular species.

2. STATE OF CHLOROPHYLL *IN VIVO*

The average concentration of chlorophyll in the photosynthetic organelles (chloroplasts or chromoplasts, dimensions $\sim 1\ \mu$) is of the order of 0·01 mole/l. Electron microscopic and optical studies suggest that the distribution of the pigment molecules in these bodies is not uniform. In many chloroplasts, the pigments are accumulated in much smaller bodies within the chloroplast, called

grana (dimensions ~ 0·1 μ): the average concentration of the pigment in the grana must be of the order of 0·1 mole/l. and the *average* distance between two neighbouring pigment molecules in the grana, of the order of 50 Å. Experimental and theoretical studies of resonance transfer of excitation energy led to the conclusion that, for strongly absorbing molecules, in which the lifetime of excitation is of the order of 10^{-8} sec, the probability of excitation energy being transferred to an adjoining molecule during the lifetime of excitation is ~ 50 per cent for a distance of about 50 Å. In other words, resonance energy transfer between pigment molecules in a granum should be a fairly probable event, even if the chlorophyll molecules were distributed in the granum at random. In fact, however, their distribution is far from uniform. The chemical nature of chlorophyll speaks in favor of its molecules being localized on the interfaces between the more hydrophilic and the more hydrophobic lipide-protein lamellae existing in the chloroplasts; and optical data support this hypothesis. The lamellae form dense stacks in the grana, but extend, in a looser structure, throughout the granular chloroplasts (Fig. 1(a)); while non-granular chloroplasts are lamellar throughout (Fig. 1(c)). Blue-green algae and purple photosynthetic bacteria, which may have no chloroplasts at all, nevertheless contain lamellae of the same general type (Fig. 1(d), (e)).

It has been calculated by Thomas *et al.* (1956) that the number of chlorophyll molecules in fully pigmented cells of various plant species is just enough to cover all lamellae by a packed monomolecular layer, allowing about 1 mμ^2 for each molecule.

It remains uncertain whether in granular chloroplasts, the intergranular lamellae are bare or also carry a monomolecular layer of the pigment. More importantly, it is at present a matter of pure speculation what is the relative location, in the lamellar structure, of the different kinds of chlorophyll (*a*, *b*, *c*), and of the accessory pigments, carotenoids and phycobilins. The carotenoids are more lipophilic than the chlorophylls and the phycobilins more hydrophylic; whether this means that they aggregate in two different phases, rather than take part in the building-up of a pigment monolayer between them, we do not know. There is, however, ample evidence that, as a general rule, the molecules of the different pigments are close enough (< 100 mμ apart) to permit a more or less efficient resonance exchange of excitation energy between them.

Figure 1 shows typical structures of chloroplasts as observed (a) in the leaf of *Zea mais* (Vatter, 1956); (b) in *Chlorella pyrenoidosa* (Albertson & Leyon, 1954); (c) in a young shoot of *Aspidistra elatior* (Leyon, 1954); (d) in *Synechococcus cedrorum* (a blue-green alga) (Elbers *et al.*, 1957); and (e) in *Porphyridium cruentum* (a red alga) (Brody & Vatter, 1959).

Attempts have been made to determine the structure of the lamellae in more detail, but the proposed structures remain controversial. Often, individual lamellae have been observed to consist of two leaves, of about 80 Å thickness each, with a space between them: the typical total thickness of a lamella is of the order of 200 Å in the dry, fixed state, and perhaps 30 per cent thicker in the wet, natural state.

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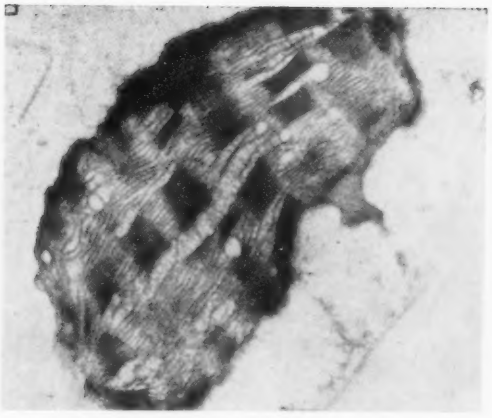

FIG. 1 (a). Electron micrograph of a section through a granular chloroplast of *Zea mais*. Vatter (1956).

FIG. 1 (b). Electron micrograph of a section through the green alga *Chlorella pyrenoidosa*. Albertson & Leyon (1954).

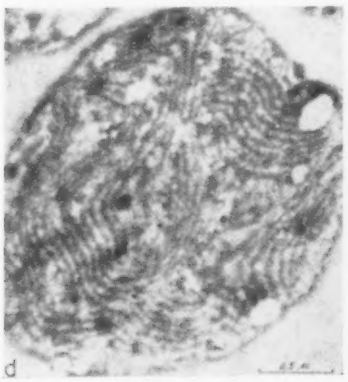

FIG. 1 (c). Electron micrograph of a section through a lamellar chloroplast in a young shoot of *Aspidistra elatior*. Leyon (1954).

FIG. 1 (d). Electron micrograph of a section through a cell of the blue-green alga *Synechococcus cedrorum*. Elbers *et al.* (1957).

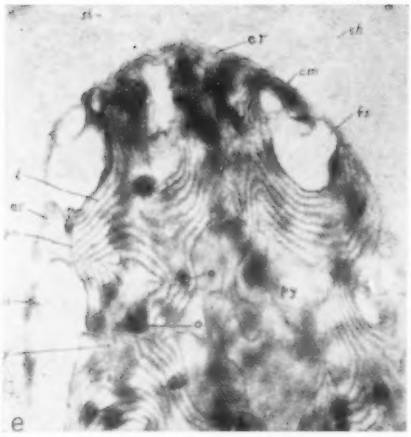

FIG. 1 (e). Electron micrograph of a section through a cell of the red alga *Porphyridium cruentum*. Brody & Vatter (1959).

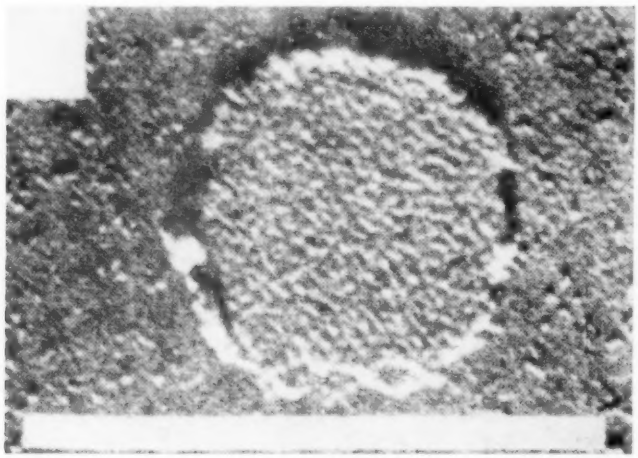

FIG. 2. Electron micrograph of the surface of a lamella from a chloroplast granum. Steinmann (1952).

Extreme amplifications of electron microscope pictures of single lamellae suggest that they consist of giant globular molecules; the surface is similar to that of a stone pavement (Steinmann, 1952). Each of these stones (cf. Fig. 2) has an area of the order of 200 $m\mu^2$ so that one can imagine it as carrying up to 200 pigment molecules. When red or blue-green algae are extracted with water, the water-soluble phycobilins come out in the form of chromoproteid molecules with molecular weights of about 250,000, each carrying 50–100 pigment molecules. The chlorophyll attachment to protein molecules may be similar, except that the complex is attached also to some lipophilic cell constituents and therefore insoluble in water.

3. EXCITATION ENERGY MIGRATION *IN VIVO*

When a quantum of light is absorbed by a molecule in the pigment-covered lamellar system, it has a chance of being transferred, by resonance—and, perhaps, repeatedly transferred—from one pigment molecule to another neighboring one, until it is re-emitted as fluorescence, dissipated into heat, or utilized to initiate a photochemical reaction. Before this happens, there is a possibility of excitation being transformed (either in the original absorbing molecule or in some molecule it visits in the transfer chain) from the initial short-lived "fluorescent" type to a non-fluorescent "metastable", usually "triplet" type. As shown by Terenin and co-workers (Terenin, 1951, 1959; Terenin & Ermolaev, 1956a, b; Ermolaev & Terenin, 1959), energy migration can be continued in this state. The longer lifetime of the metastable state ($>10^{-5}$ sec) gives increasing opportunity for its migration but, on the other hand, coupling between two resonating molecules is infinitely weaker in the metastable than in the unstable, "fluorescent" state. Consequently, it is doubtful whether migration in the metastable state can increase significantly the overall range of energy migration in a closely packed pigment system, such as a chlorophyll-coated lamella.

Two types of energy migration can be distinguished, which could be called *homogeneous* and *heterogeneous*, respectively. "Homogeneous" migration implies identity of the energy donating and the energy accepting molecule: it can be repeated again and again. In photosynthesis, we are concerned with the possibility of such migration mainly between molecules of chlorophyll *a* which is the major component of the pigment mixture in most photosynthesizing cells. As shown by Förster (1951, 1959), the probability of resonance energy migration depends on the overlapping of the emission band of the energy donor molecule and the absorption band of the energy acceptor molecule. This overlapping often is larger between two different than between two identical molecules, because the fluorescent band is often situated at considerably longer wave lengths than the absorption band of the same molecular species. In the case of chlorophyll *a*, however, these two bands are close and strongly overlap each other (Fig. 3), so that a chlorophyll layer is more apt to support homogeneous energy migration than layers of most other pigments: conceivably, this may be significant for nature's choice of chlorophyll as the main sensitizing pigment in photosynthesis.

The band overlapping is very strong between the two chlorophylls *a* and *b*: the absorption band of chlorophyll *a* lies at 660 mμ (in ether) while the emission band of chlorophyll *b* (in the same solvent) lies at 657 mμ. It is therefore reasonable

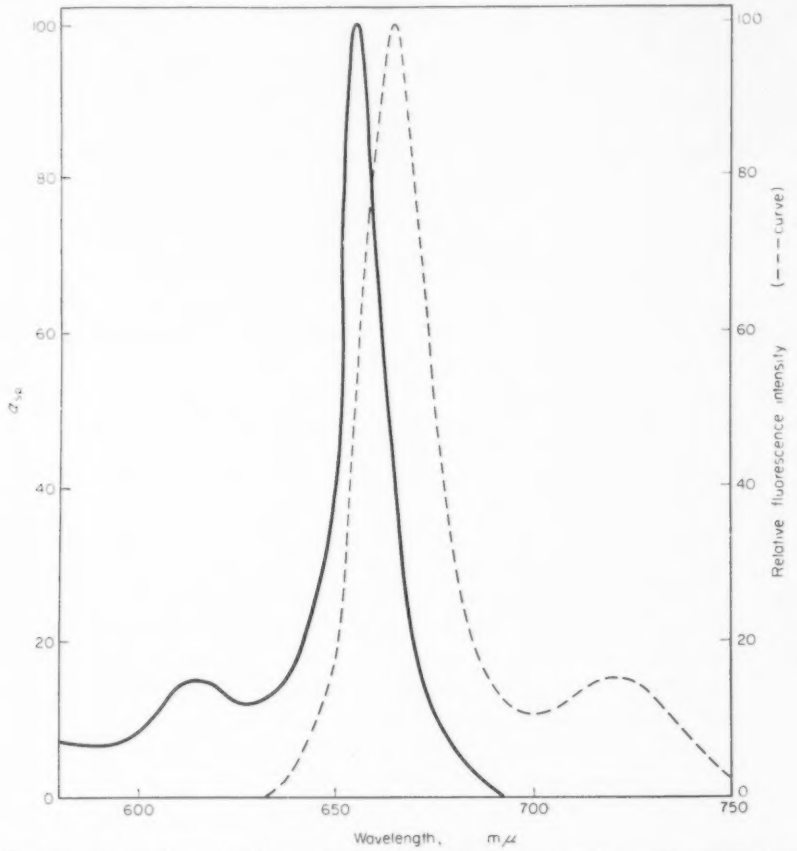

FIG. 3. Absorption and fluorescence of chlorophyll *a* in ether. (Redrawn after Zscheile *et al.* (1941, 1943).)

to suppose that light energy absorbed *in vivo* by chlorophyll *b* will be transferred with very high efficiency to chlorophyll *a*. The reverse transfer—from *a* to *b*—is practically impossible, because it would require the addition of a considerable amount of thermal energy to the energy of excitation. In general, heterogeneous energy transfer occurs practically in one direction only—from molecules having absorption bands farther towards the violet, to molecules having absorption bands farther towards the red. Therefore, in the natural mixture of photosynthetic pigments, energy absorbed by carotenoids could be transferred to phycoerythrin, the excitation energy of phycoerythrin to phycocyanin, thence to chlorophylls *c* or *b*, and ultimately to chlorophyll *a*.

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It will be noted that, in order to serve as an efficient energy acceptor, a pigment does not need to be present in large amounts. This amount can be insufficient for a significant direct absorption of light, but enough to act as an efficient trap for migrating energy quanta originally absorbed by other pigments. For example, it has been suggested by Kok (1956, 1957) that photosynthesis may be brought about by a pigment, present in very small amounts, but having an absorption band on the long-wave side of that of chlorophyll *a* (at 705 mμ). Excitation energy of a large number of chlorophyll *a* molecules could migrate through the pigment layer until it falls into the traps provided by an occasional molecule of this rare pigment. Such a process has been observed by French & Young (1952) and by Duysens (1952) in some red algae where a small amount of an unknown pigment (perhaps chlorophyll *d*) "robs" chlorophyll *a* of almost all of its excitation energy, using it for the emission of its own fluorescence.

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4. EVIDENCE OF HOMOGENEOUS ENERGY MIGRATION

The best evidence of homogeneous energy migration in plant cells material is provided by observations of the depolarization of fluorescence in phycobilins. Unless all molecules in a pigment aggregate are arranged in parallel, every energy transfer must lead to a change in the direction of the oscillating electric dipole responsible for the emission of fluorescence. After a few such transfers, all preference for parallelism between the electric vectors of the absorbed and of the emitted light will be lost. If excitation is produced by plane-polarized light, fluorescence normally is polarized too; but if the energy migration occurs, fluorescence may be more or less completely depolarized. This is exactly what has been observed by Goodheer (1957) in the fluorescence of phycobilins, even when observed in molecular dispersion. This can be interpreted as evidence that a quantum absorbed by one of the pigment molecules attached to a given protein molecule in the chromoproteid migrates through other pigment molecules before it is emitted as fluorescence. It is plausible to postulate that something similar does happen also with excitation quanta absorbed *in vivo* by chlorophyll: but systematic investigations are missing which would permit us to determine the extent of homogeneous energy migrations in the chlorophyll layers of photosynthesizing plant cells. In particular, we do not know whether this migration is sufficient to explain the kinetic data which suggest the existence of a "photosynthetic unit"—a body containing 200–300 chlorophyll *a* molecules associated with a single enzyme molecule. (As mentioned above, the giant protein molecules observed in the electron micrograms of lamellae may be able to accommodate up to 200 pigment molecules.)

Energy migration can lead not only to *depolarization*, but also to complete *quenching* of fluorescence, if, in its course, the energy quantum encounters molecules or molecular complexes in which the excitation energy can be easily dissipated—either by conversion into heat, or by utilization for a photochemical reaction. The fluorescence yield of chlorophyll *a* in solution is of the order of 30 per cent; i.e. out of ten absorbed quanta, three are re-emitted. In the living cell, the yield is reduced

to 2–3 per cent, and depends on the intensity of illumination and on the previous history of the cell. It seems not unlikely that this reduction in fluorescence yield is due to energy migration between the chlorophyll molecules *in vivo* which ends in an encounter with an energy-dissipating molecular complex. The dissipation process may be the primary photochemical process of photosynthesis itself.

5. EVIDENCE OF HETEROGENEOUS ENERGY TRANSFER

More ample and quantitative evidence exists on the subject of *heterogeneous* energy transfer. The latter reveals itself by the occurrence of *sensitized fluorescence*; light quanta absorbed by one pigment are emitted as fluorescence of another pigment, easily distinguishable by its different position in the spectrum. The occurrence of sensitized fluorescence in photosynthesizing cells had been first discovered by Dutton *et al.* (1943) in the diatom *Nitzschia*, where light quanta absorbed by the carotenoid, fucoxanthol (responsible for the brown color of the algae), were found to produce the fluorescence of chlorophyll *a* with approximately the same yield as light absorbed by chlorophyll itself. Duysens (1952) studied quantitatively the sensitized fluorescence of chlorophyll in algae of various divisions, and of bacteriochlorophyll in photosynthetic bacteria, and arrived at "efficiency factors" for heterogeneous energy transfer listed in Table 1.

TABLE 1—EFFICIENCY OF ENERGY TRANSFER

From	To	% of excitation quanta transferred
Chl *b*	Chl *a*	96
Phycocyanin	Chl *a*	>90
Phycoerythrin	Phycocyanin	>80
Fucoxanthol (and other carotenoids in diatoms)	Chl *a*	~70
Carotenoids (in green algae)	Chl *a*	40–50
BChl 850	BChl 900	100
BChl 800	BChl 850	100
Carotenoids	BChl	40

The figures in Table 1 should not be taken as precise. As shown by Brody & Emerson (1959), the efficiency of energy transfer from phycoerythrin to chlorophyll *a* in the red algae depends on their pre-illumination with the light absorbed by phycoerythrin (which seems to improve the coupling of phycoerythrin with chlorophyll *a*) or by chlorophyll itself (which seems to produce a de-coupling).

It seems plausible that the effective contribution to photosynthesis of light quanta absorbed by fucoxanthol in brown algae, by the phycobilins in red and blue-green algae, and by chlorophyll *b* in green plants, is due to highly efficient transfer of their excitation energy to chlorophyll *a*.

6. PHOTOCONDUCTION—A STEP IN PHOTOSYNTHESIS?

The possibility has been suggested—first by Katz (1949), and more recently by Arnold & Sherwood (1957, 1960) and Calvin & Sogo (1957) and Tollin *et al.* (1958)—that another of the solid-state phenomena mentioned in the introduction plays a role in photosynthesis, namely, the conversion of an excited molecular electron into a "free" electron, which can move through the solid phase (while the remaining "hole" can also move about by repeated electron replacement from neighboring molecules). Fig. 4 illustrates the difference between this process and the above-considered resonance energy migration. The term "exciton", first suggested by Frankel (1931, 1936), is often used to describe both processes. Exciton is the pair electron-hole. Resonance energy migration can be described as the movement of an "intramolecular exciton"—the electron and the hole remaining in the same molecule (Fig. 4(a) or (b)): it migrates without separation of charges. "Intermolecular exciton", on the other hand, involves a separation of the positive from the negative charge (Fig. 4(c)); both remain in the solid but become separated

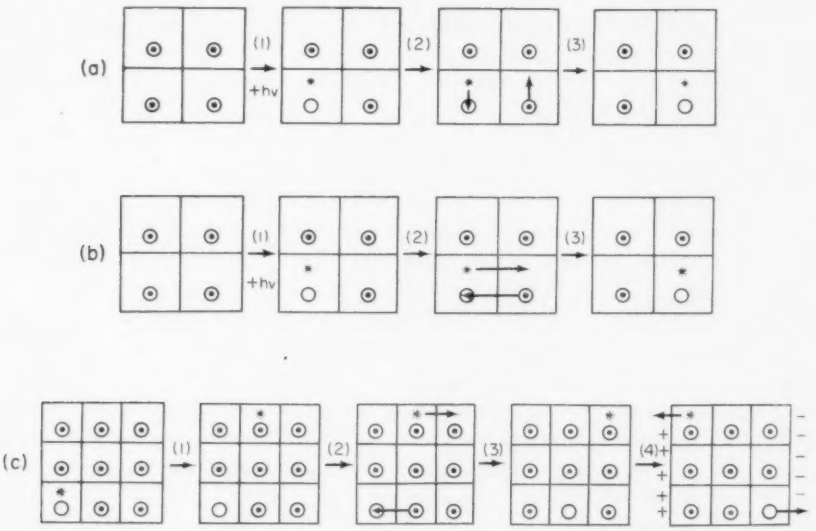

FIG. 4. Schemes of exciton migration. (a) Intramolecular excitation, formation (1), and migration (2, 3). Forster–Heller mechanism. (b) Same, Wannier mechanism. (c) Intermolecular exciton formation (1), migration (2, 3), and dissociation (4).

by distances much larger than a molecular diameter. Under the influence of electric forces, such an exciton may "dissociate", and the charges migrate out of the system. It is the latter possibility that seems attractive to some students of photosynthesis. Dissociation of an intermolecular exciton, and separate migration of the two charges through the chloroplast layer, could conceivably lead to the primary reduction process (transfer of the electron to a reactant) to occur in a site

separated from that of the primary oxidation process (electron acceptance from another reactant). This ready separation of the two primary reaction products could prevent their immediate back reaction ("primary re-combination") and thus help to explain the high quantum yield of photosynthesis. (Under favorable conditions, practically every absorbed quantum of light is utilized in this process!)

Several observations have been quoted in support of this picture. An electron made "free" in the crystal lattice (be it by heating, or by light absorption) often ends by being trapped in a potential minimum that can be provided by a lattice dislocation or an electropositive impurity. These trapped electrons can be released from the traps by the absorption of relatively small light quanta—often such of infra-red light—or by heat, returning back into the "free" state. They can then recombine with the "holes" they had left behind, emitting phosphorescence. Furthermore, while the electrons are in the "free" state, they can follow an outside electric field, applied to the crystal: they thus produce photoconductivity. All these phenomena have been observed in dried chloroplast films. Furthermore, paramagnetic resonance (suggestive of the presence of free electrons, or radicals) has been found in the same plant material (Commoner, 1956) (as well as in crystalline chlorophyll (Brody, 1960)).

It is, however, uncertain whether this behavior is characteristic of chlorophyll in the live cell. Upon drying, local sintering or even crystallization can occur, creating solid-state properties which were not present before. Furthermore, the quantum yield of the formation of free (or trapped) electrons in illuminated chloroplast films seems to be low; because of the high yield of the primary process in photosynthesis, only those phenomena can be considered as relevant to the mechanism of photosynthesis which can occur with a high quantum yield. Finally, the assumption that electron transfer into a "conductance level" is a step in photosynthesis is contradicted by the shape of the adsorption spectrum of chlorophyll *in vivo*. When the excitation levels of *individual* molecules are replaced, in a condensed phase, by a *common* conductance level, this results in the replacement of the relatively narrow absorption band characteristic of free molecules by a much broader (and strongly displaced) absorption band of the crystal as a whole. Nothing of this kind happens to the red absorption band of chlorophyll in the chloroplasts. True, it is shifted towards the longer waves, but by only 10 to 15 mμ from its position in molecular solutions, and its broadening is not as strong as one would expect for a conductance band.

Rosenberg (1958, 1959) suggested that photoconductivity can appear without much change in the absorption band, if the conductance level arises not from the interaction of the unstable, "fluorescent", excited states of the individual molecules, but from the fusion of the metastable "triplet" states. According to this picture, absorption in the red chlorophyll band *in vivo* would lift chlorophyll into excited states belonging to individual molecules from the fusion of the triplet metastable levels. This hypothesis does not seem plausible because of the generally weak interaction between the triplet states. Furthermore, in the case of chloroplast films, Rosenberg (personal communication) found that their photoconductivity has

a marked temperature coefficient, while the quantum yield of the primary photochemical process of photosynthesis is independent of temperature.

Altogether, at the present time, resonance energy migration appears more relevant to the mechanism of photosynthesis than photoconduction. Formation of free (or trapped) electrons could well occur in illuminated chlorophyll layers as a rare event, for example, if minute areas exist in which the pigment molecules form a regular crystalline structure. That the great bulk of the chlorophyll *in vivo* is *not* in this state follows convincingly from the difference between the absorption spectrum of chlorophyll in the living cell and that of crystalline chlorophyll. Fig. 5, taken from the work of Jacobs *et al.* (1954, 1954, 1957), shows the relative

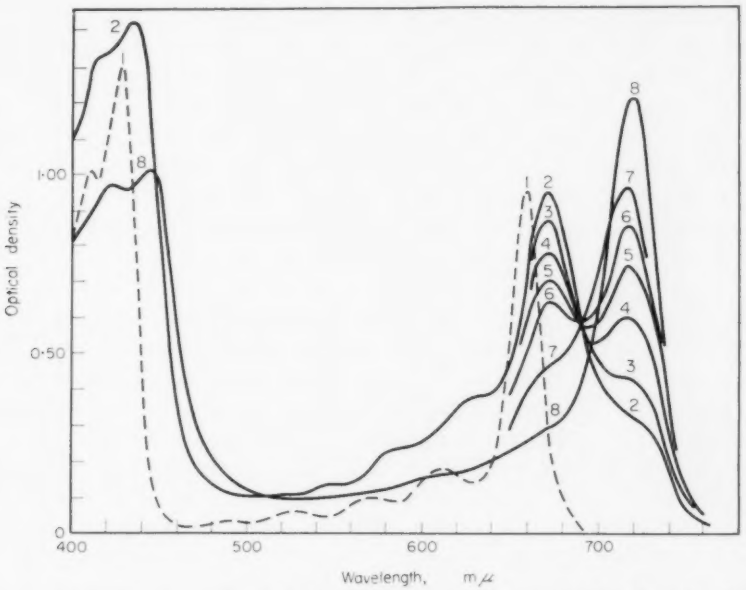

FIG. 5. Absorption spectra of molecularly dispersed (1), colloidal (2) and microcrystalline (3–8) ethyl chlorophyllide *a*. Note band shift towards longer waves with increasing size of microcrystals (710→720 mμ, from (5) to (7); limiting position, 740 mμ). Jacobs *et al.* (1954, 1954, 1957).

positions of the red chlorophyll band in true solution, in amorphous colloidal particles, and in microcrystals of different size. It reveals a displacement of the order of 70 mμ for the crystals consisting of only a few chlorophyll molecules, the absorption band should be shifted far beyond its actual position in the living cells (670–680 mμ). This does not preclude, of course, the presence of some small microcrystals, provided their total amount is too small to cause noticeable absorption in the region of 720–740 mμ. If one wants, one can even speculate that such tiny crystalline enclosures provide traps into which energy quanta, absorbed by the bulk of chlorophyll, disappear by energy migration; and that the formation of free

electrons in these traps is an essential step in photosynthesis: but I see no experimental (or theoretical) need for postulating such a mechanism.

7. PARTICIPATION OF CHLOROPHYLL IN THE PRIMARY PROCESS

The most plausible picture of the first, "photophysical", step in photosynthesis appears to involve the migration of the excitation quanta over a limited number (of the order of 100) chlorophyll molecules. These quanta could be initially absorbed by chlorophyll *a* molecules or transferred to them by resonance from auxiliary pigments. This migration ends in a pigment molecule capable of serving as an energy trap, in which excitation energy can be converted into chemical energy. One can imagine this conversion as not involving any chemical change in the sensitizer. For example, one could envisage chlorophyll associated in one point with an electron donor (reductant) and in another point with an electron acceptor (oxidant). When this chlorophyll molecule acquires a quantum of electronic excitation, it may "gulp" an electron (or H atom) in one point and give away an electron (or an H atom) in the other point, and emerge from this practically instantaneous process in its original form. The other alternative is that these two reactions do not occur simultaneously, but with an interval between them, long enough to produce, in the photostationary state, a significant amount of chlorophyll in the chemically changed state; the latter may be reduced, oxidized, or a mixture of both.

Indirect arguments in favor of the second hypothesis can be derived from observations of reversible reduction (and oxidation) of chlorophyll *in vitro*. Particularly suggestive are observations of the Krasnovsky reaction (1948a, b, 1956) in which chlorophyll dissolved in pyridine is reduced in light by ascorbic acid. It can be oxidized by such compounds as riboflavin or safranin, or by molecular oxygen. The system can be used to produce chlorophyll-sensitized oxidation-reductions against the gradient of the chemical potential—for example, the reduction of riboflavin by ascorbic acid (which cannot occur in darkness). This provides attractive models of the function of chlorophyll in photosynthesis (where it has to sensitize the reduction of carboxyl or carbonyl groups by water).

The reversible oxidation of chlorophyll by ferric ions (which occurs in the dark but is accelerated by light) observed by Rabinowitch & Weiss (1937) also may be relevant to the function of chlorophyll in nature. Photosynthesis may involve both reversible photochemical oxidation and reversible photochemical reduction, permitting the use of two light quanta for the transfer of each one of the four hydrogen atoms, which need to be moved to carbon dioxide to reduce the latter to the level of carbohydrate.

One can hope to get a more direct evidence of a possible reversible chemical change of chlorophyll in photosynthesis from observations of the co-called *difference spectrum* of photosynthesis—the difference between the absorption spectra of photosynthesizing cells in light and in darkness. Duysens (1953, 1954a, b, 1958) was looking for such a difference when he first introduced difference spectroscopy into the study of photosynthesis. However, he found only some not

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very clear changes of the bacteriochlorophyll bands in purple bacteria, and none of the chlorophyll bands in green algae. Instead, he found consistent changes which could be attributed to a reversible oxidation of cytochromes, and a reversible reduction of pyridine nucleotides—which may, or may not, be related to the primary photochemical process.

Subsequent studies by Coleman (Coleman *et al.*, 1957, 1959) and Kok (1956, 1957a, b) have shown that if stronger light is used for illumination, reversible changes in the chlorophyll spectrum do appear and ultimately become the most conspicuous features of the difference spectrum (at least in *Chlorella*). Fig. 6

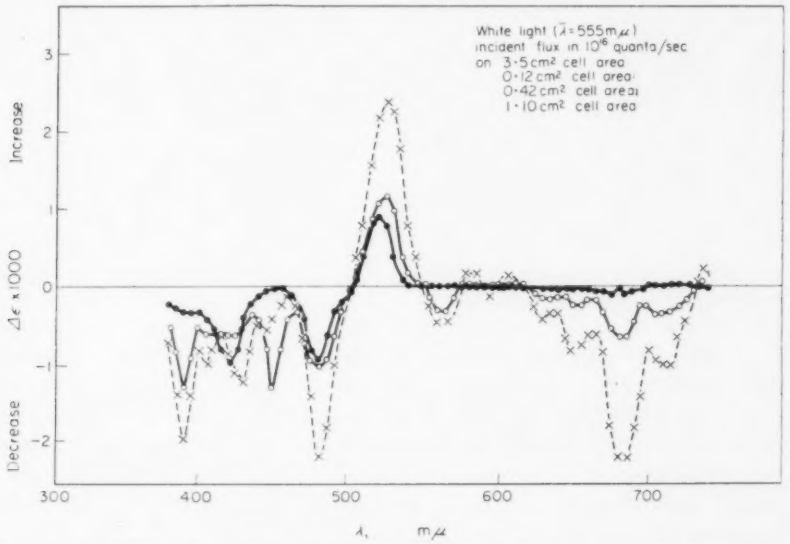

FIG. 6. Difference spectrum of a *Chlorella* suspension in carbonate buffer. White actinic light intensities: dots, $3{\cdot}3 \times 10^{14}$ quanta/(cm^2 × sec); circles, $1{\cdot}2 \times 10^{15}$ quanta/(cm^3 × sec); crosses, $3{\cdot}1 \times 10^{16}$ quanta/(cm^3 × sec). Coleman *et al.* (1957, 1959).

shows the difference spectrum of *Chlorella* according to Coleman *et al.* (1957, 1959) for four different intensities of illumination. The reversible transformation of chlorophyll *during* photosynthesis (and presumably associated *with* photosynthesis) shows itself by the bleaching at 440 and 680 mμ in the two main peaks of the chlorophyll *a* spectrum (420 and 660 mμ in solution). The other striking change (which can be observed already in weaker light) is a bleaching at 480 mμ and increased absorption at 515 mμ, which may conceivably be due to a reversible transformation of a carotenoid. This latter effect makes it difficult to decide whether the bleaching at 680 mμ is accompanied by an increase in absorption at 520 mμ, which should be the case if bleaching were due to a reversible reduction of chlorophyll *a* to "eosinophyll"—the pink reduced form of chlorophyll found by Krasnovsky.

The fact that the bleaching at 680 mμ does not occur in weak light, and follows a sigmoid curve rather than being proportional to light intensity (Fig. 7), could be explained by assuming that as long as the enzymatic follow-up processes keep step

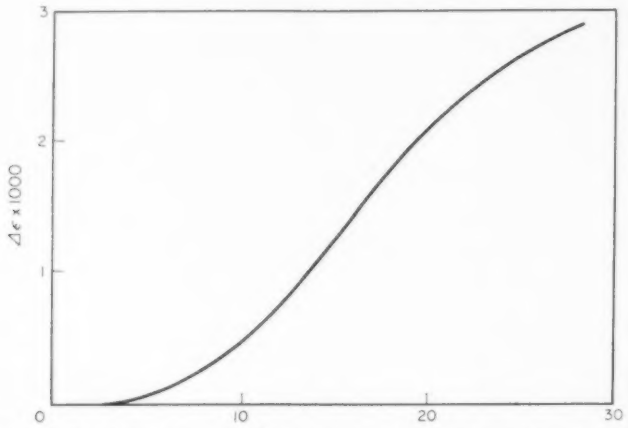

FIG. 7. Reversible bleaching at 680 mμ as function of intensity of actinic light. *Chlorella* suspension in carbonate buffer. Coleman & Rabinowitch (1957, 1959).

with the primary photochemical process, the reversible reduction of chlorophyll does not go beyond an intermediate free radical, and is almost instantaneously reversed. Only when the enzymatic steps fall behind the primary photochemical process, do chlorophyll radicals begin to accumulate in a concentration sufficient to permit their conversion (by dismutation) into the more stable, fully reduced eosinophyll, and bleaching becomes noticeable. Simultaneously, the quantum yield of photosynthesis declines and light saturation sets in.

This must remain a plausible speculation as long as the absorption growth at 520 mμ cannot be clearly correlated with its decrease at 680 mμ.

The form of the difference spectrum in the region of 640–700 mμ is of interest. Its complexity indicates that the several components of the red chlorophyll band *in vivo* are not affected equally.

All these matters will be ripe for interpretation when the measurements will be much more systematic and extended to a greater variety of plant material. Rubinstein's new data (unpublished) clearly indicate that the curves in Fig. 6 are distorted by superposition of a practically instantaneous change (which is practically instantaneously reversible) over a comparatively slow change which is equally slowly reversed in the dark. At a given wavelength, the two effects may have the same or the opposite sign—for example, at 550 mμ, the immediate effect is increased absorption, and the slow effect is bleaching.

At present, all that can be said is that the studies of the difference spectrum make the hypothesis of a reversible chemical change of chlorophyll in photosynthesis more plausible, without definitely proving it.

8. SEVERAL CHLOROPHYLL *a* FORMS *IN VIVO*

The picture of the primary photochemical process in photosynthesis has been made more interesting—and more complicated—by recent experiments showing that chlorophyll *a* is present, in the living cell, in at least two (and probably more) different forms, having different photochemical functions. It has been noted long ago that the width of the red chlorophyll band *in vivo* suggests a complex structure. Absorption curves of green cells or chloroplast suspensions often appear to have several maxima (Albers & Knorr, 1937). Analysis of these bands by means of French's derivative spectrophotometer confirmed this complex structure (French & Brown, 1959). Finally, the precise integrating spectrophotometer, developed by Cederstrand (unpublished), gave direct evidence of the presence of two or more peaks in the absorption bands of several types of algae, as illustrated by Fig. 8.

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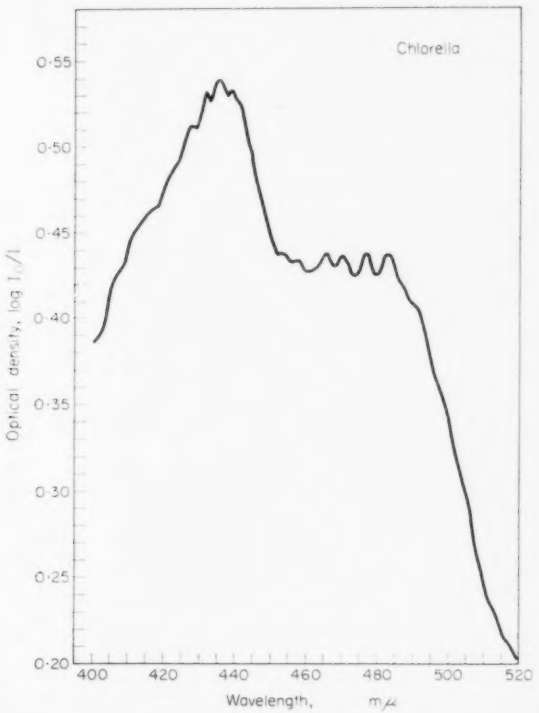

FIG. 8(a).

FIG. 8. Absorption spectra of several unicellular algae measured with an integrating spectrophotometer (12 photocells on 12 sides of a dodecahedron). Note multiplicity of main bands. Cederstrand (unpublished).

This suggests an interpretation of some striking results obtained in the determination of the action spectra of photosynthesis initiated by Emerson and

co-workers (Emerson *et al.*, 1943; Tanada, 1951). Emerson & Lewis (Emerson *et al.*, 1943) first noted in 1942 the drop of the quantum yield of photosynthesis in *Chlorella*, above about 680 mμ, well within the absorption band of chlorophyll *a*.

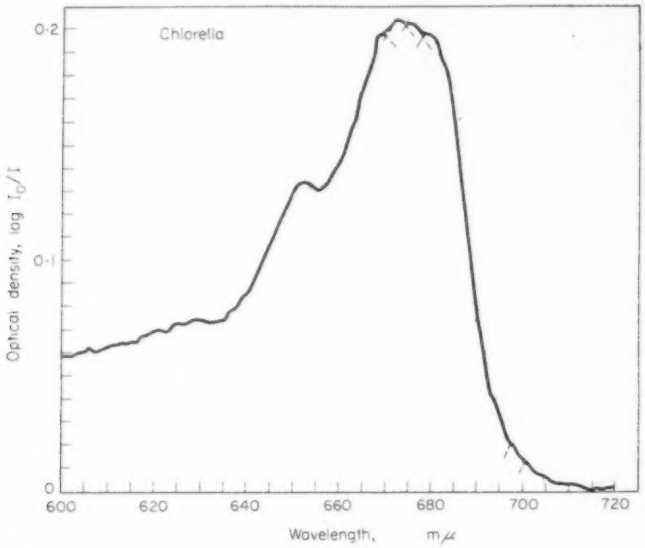

FIG. 8(b).

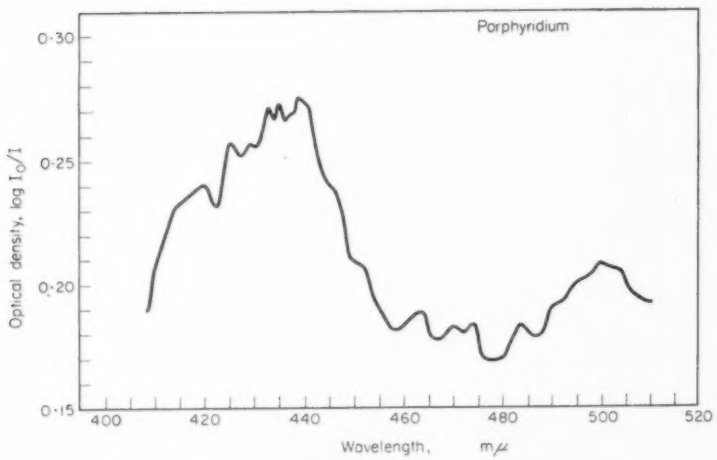

FIG. 8(c).

In 1950, Haxo (1950) noted a much more striking decline in the yield of photosynthesis in red algae, in the main chlorophyll absorption region in the red. They

interpreted this observation as evidence of a relative inefficiency of chlorophyll *a* as sensitizer of photosynthesis in these organisms. However, Brody & Emerson (1959) concluded, from a more precise study, that what actually happens is that the

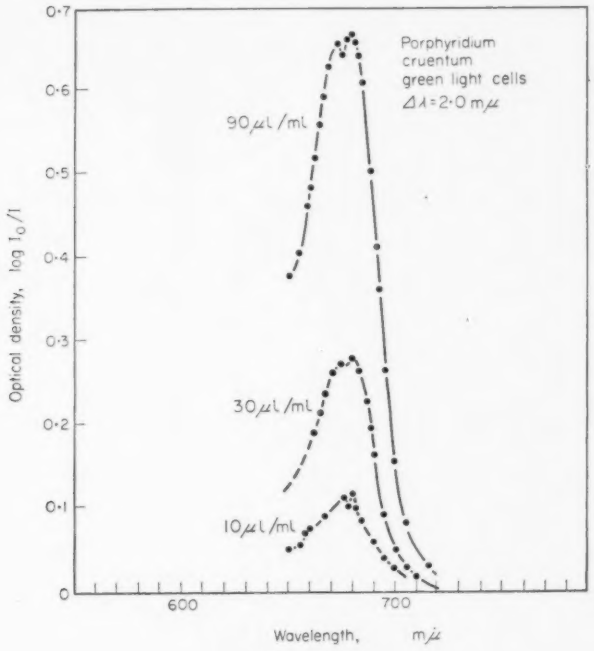

FIG. 8(d).

"red drop" begins in these algae already at 650 mμ (instead of at 680 mμ, as in *Chlorella*). Chlorophyll *a* absorption below 650 mμ is, in red algae, as efficient as (or more efficient than) the absorption of phycobilin pigments.

More recently Emerson *et al.* (1956) and Emerson *et al.* (1958, 1960) found that the low photosynthetic efficiency of light in the region of the red drop can be enhanced, and often brought up to normal, by simultaneous absorption of light of the shorter wave lengths. The action spectrum of this "second Emerson effect" was found to follow the curve showing the percentage of total absorbed light, absorbed by certain auxiliary pigments—chlorophyll *b* in *Chlorella*, fucoxanthol in diatoms, and the phycobilins in red and blue-green algae (Fig. 9). A tentative conclusion was drawn that, in addition to the excitation of chlorophyll *a*, photosynthesis requires excitation of one of the auxiliary pigments; the "red drop" occurs when chlorophyll *a* becomes the *only* absorber. This happens in red algae (which contain no chlorophyll *b*) at much shorter wave lengths than in chlorophyll *b*—bearing green algae.

This picture, while consistent in itself, contradicted the conclusions drawn from the observations of sensitized chlorophyll fluorescence (cf. Section 5). The latter indicated that light absorbed by auxiliary pigments is transferred, more or less effectively (in case of chlorophyll *b*, with practically 100 per cent efficiency), to

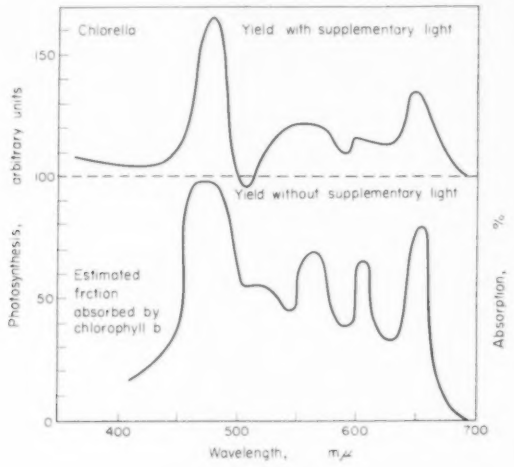

FIG. 9(a).

FIG. 9. Action spectra of the Emerson effect in different divisions of algae. Note "negative" effects (curves dipping below 100). Emerson *et al.* (1958, 1960).

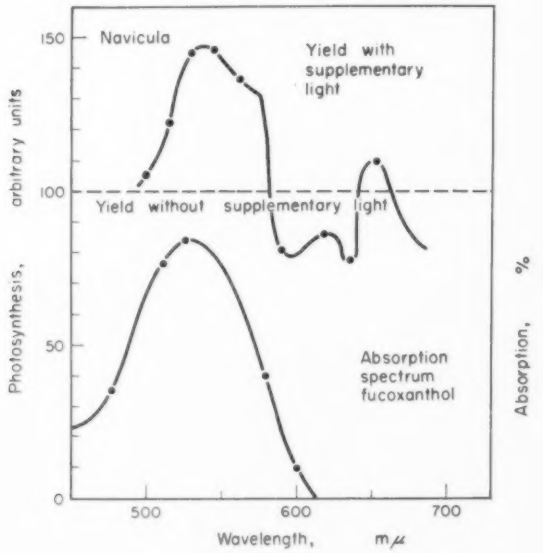

FIG. 9(b).

chlorophyll *a*; and that the efficiency with which light absorbed by an auxiliary pigment contributes to photosynthesis is determined by the efficiency with which its excitation is transferred to chlorophyll *a*.

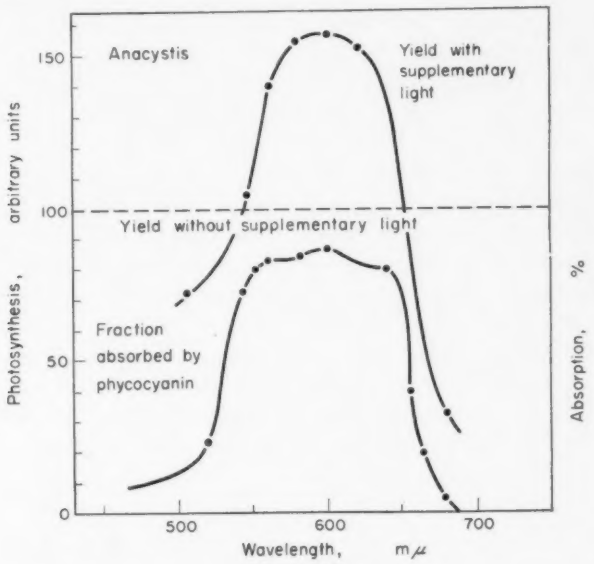

FIG. 9(c).

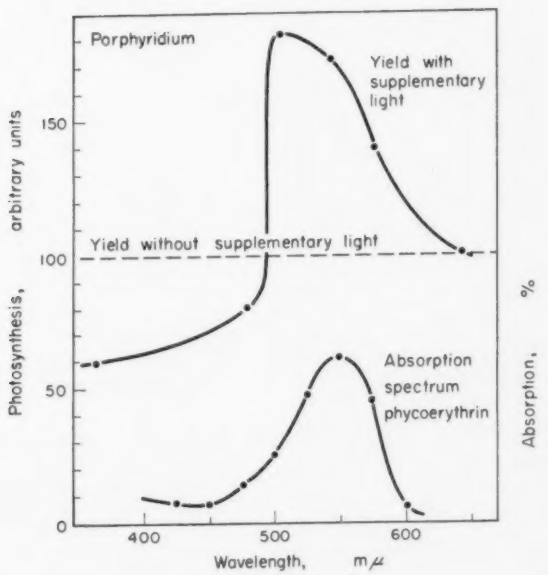

FIG. 9(d).

9. CHLOROPHYLL *a* 670 IN THE EMERSON EFFECT

Franck (1958) suggested that the solution of this paradox should be sought in the existence in the cell of two types of chlorophyll *a*, which must both be excited, in a balanced way, to permit the primary process of photosynthesis to proceed effectively. Because of the directional energy flow from pigments absorbing at shorter waves to those absorbing at longer waves, the required balancing of excitation can occur automatically: when the chlorophyll *a* component absorbing at the shorter wave lengths is excited, it shares its excitation, by resonance transfer, with the component absorbing at the longer waves. Exclusive excitation of the component that absorbs at the longer waves, on the other hand, cannot be similarly shared, and this is the reason for the red drop. Franck based this interpretation of Emerson's result on considerations in a different field, which have led him already in 1958 to postulate the need for a two-fold primary photochemical process in photosynthesis, requiring two forms of chlorophyll *a* to bring them about—one form present in an entirely lipophilic environment, and one associated with water.

Recently, Govindjee *et al.* (1960a, b) gave direct evidence of the correctness of Franck's interpretation. They found that, in addition to the bands due to auxiliary pigments, the action spectrum of the Emerson effect also contains a band at 670 mμ (cf. Fig. 10) which must be due to a chlorophyll *a* component—obviously

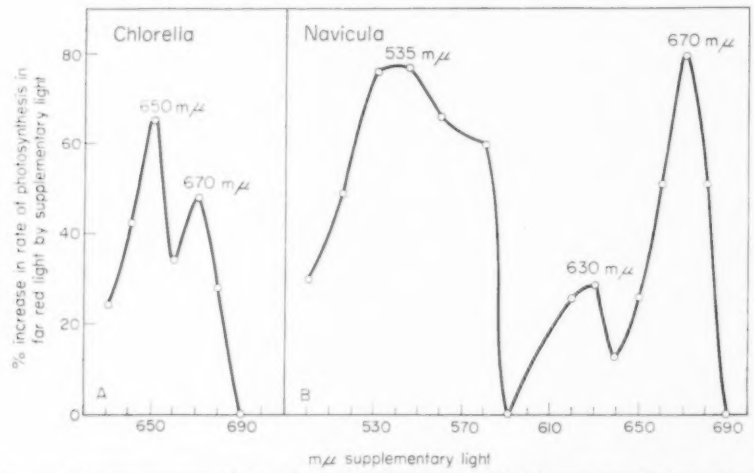

FIG. 10. Action spectra of the Emerson effect taken with better resolution, showing the 670 mμ band due to a form of chlorophyll *a*. Govindjee *et al.* (1960a, b).

the same one as revealed by the 670 mμ absorption peak in Cederstrand's absorption measurements (cf. Fig. 8).

It is a speculation—but a plausible one—that light absorption by auxiliary pigments produces the Emerson effect by preferential transfer of their excitation energy to chlorophyll 670 mμ (as one would expect from the relative position of the absorption bands).

10. LIGHT SATURATION IN FAR RED LIGHT

It was noted by Govindjee (1960b), Govindjee (in preparation) and by French & McLeod (1960) that photosynthesis produced by far red light (in the region of the red drop) not only has a lower quantum yield, but is also saturated earlier and at a much lower level than photosynthesis produced by light of shorter wave length. Fig. 11 illustrates this unexpected behavior. It is unexpected because, according

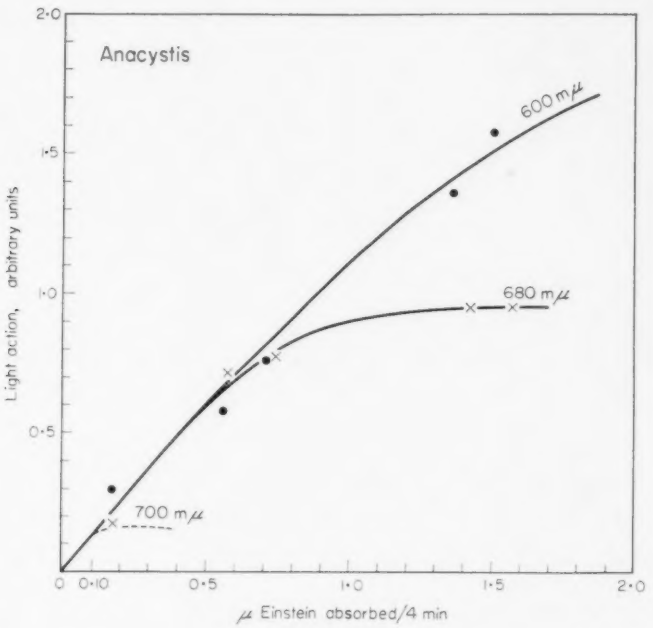

FIG. 11(a).

FIG. 11. Light saturation curves of blue-green, green and red algae in monochromatic light, showing earlier saturation in far red light. Govindjee *et al.* (1960b) and Govindjee (in preparation).

to the generally accepted picture of the basic kinetics of photosynthesis, light saturation occurs (in the presence of sufficient carbon dioxide) when the rate of an enzymatic reaction reaches its maximum possible value; and there is no reason why this rate limitation should depend on the quality of light producing the primary photochemical process. Gabrielson (1940) found, in fact (in the only experiments of this type described in the literature), that the saturation level of photosynthesis is the same in three parts of the visible spectrum (red-orange, yellow-green and blue-violet) (Fig. 12). Photosynthesis produced by far red light must involve a different limiting dark reaction, with a lower maximum rate.

The question arises whether the two features observed in extreme red light—low quantum yield and low saturation level—are two independent phenomena.

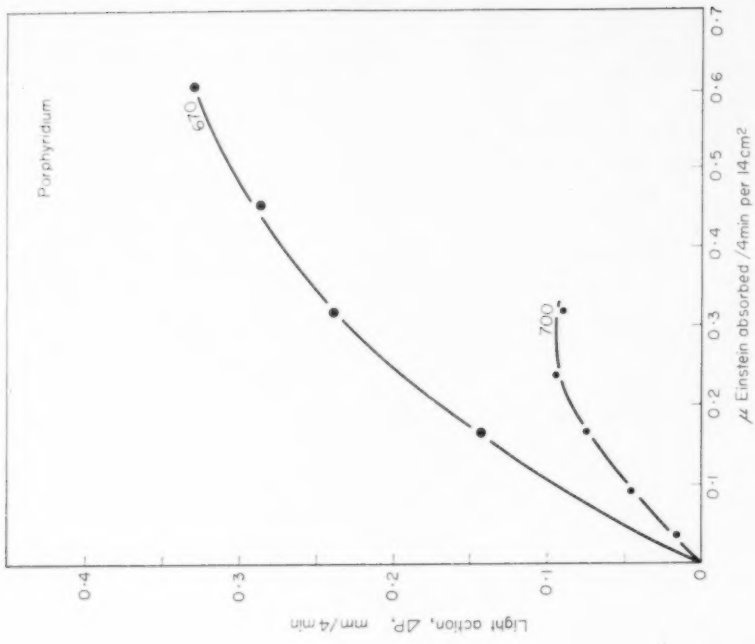

FIG. 11(c).

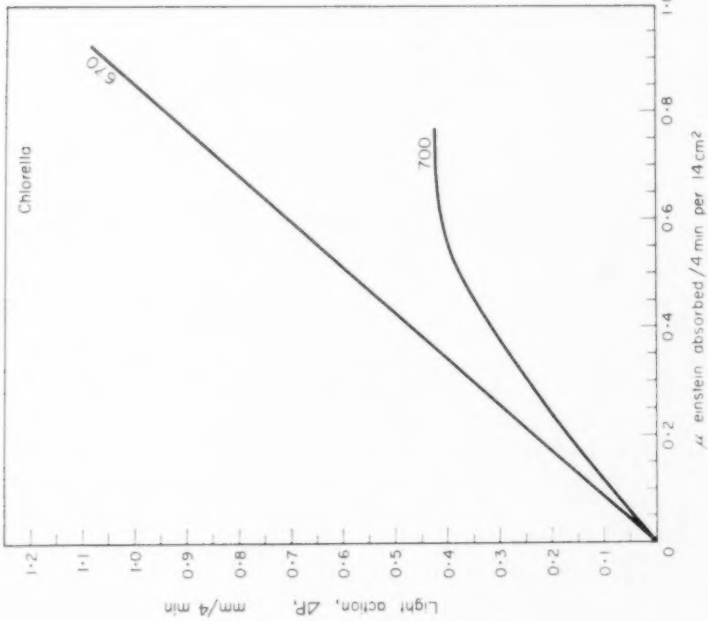

FIG. 11(b).

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One could imagine, for example, that the low quantum yield is merely an "advance notice" of the low saturation level; as discussed elsewhere (Rabinowitch, 1951) the influence of an approach to a saturation plateau extends far below the actual saturation rate. However, this would require the light curves to show a marked curvature from the very beginning, while Emerson satisfied himself that he was working in the practically straight segment of the light curve for far red light.

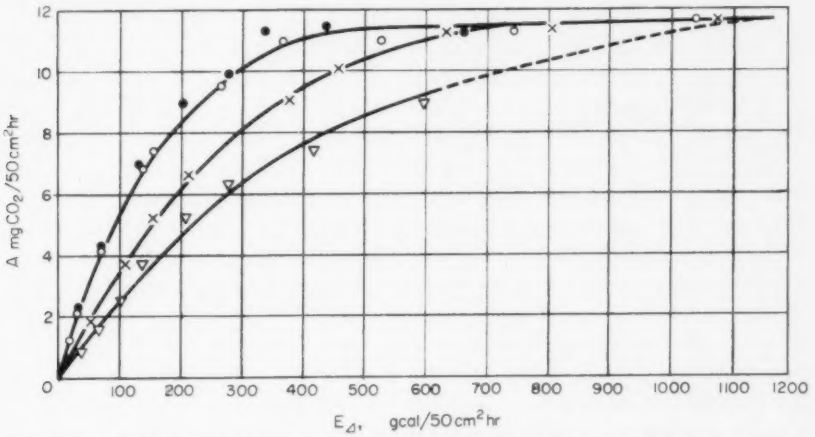

FIG. 12. Light saturation curves of *Sinapis alba* showing identical saturation levels in blue-violet light (triangles), yellow-green light (crosses) and orange-red (circles). Gabrielson (1940).

Furthermore, preliminary measurements by Govindjee of light curves of photosynthesis in monochromatic light indicate great variability, with some curves showing steep initial slope (high quantum yield), but early and relatively low saturation yield are two aspects of the same phenomenon. These experiments clearly suggest the need for measuring the complete monochromatic light curves of photosynthesis as systematically as Emerson and his successors have measured the initial slopes of these curves, then proceeding with the measurement of light curves in combined beams of different wave length, etc. The "red drop" and the Emerson effect are likely to prove, in such systematic study, to be only one aspect of a much more complex picture. The "inactive" chlorophyll (Chl 680–690) is bound to have absorption bands not only at 680–700 mμ, but also in other parts of the spectrum; in fact, preliminary examination of the Soret band *in vivo* (cf. Fig. 8(a), (c)) reveals a similar complex structure: and this factor alone should be enough to produce individual variations in quantum yield and light saturation, even if only light absorbed by chlorophyll pigments is taken into consideration.

11. NEGATIVE EMERSON EFFECT

Already Emerson's curves of the enhancement effect (Fig. 9) revealed occasional occurrence of a "negative" effect in some algae in certain spectral regions; the

quantum yield was *lower* in the combined light than in either of the two beams separately. Govindjee's studies (1960, 1961) revealed that this reversal of the sign of the Emerson effect occurs quite generally, whenever the far red light is sufficiently strong; both its absolute intensity and the ratio of intensities of the two beams are of importance.

Another question in need of systematic study is that of the difference between the red and the green algae in the onset of the red drop. The red algae, too, appear to contain the component Chl *a* 670 (Fig. 8). The absorption of light in the region 650–680 mμ, where the phycobilin absorption disappears, should be due mainly to this chlorophyll variety; but the drop in the quantum yield suggests that this is not the case. Perhaps the participation of the "inactive" chlorophyll (Chl 680–690) in the absorption in this region is greater than expected. For example, the "Chl 680" absorption band could be double, with a major component on the long-wave side of the Chl 670 band, and a minor component on the short-wave side of the latter. This latter could be of no great significance in the presence of chlorophyll *b*, which absorbs strongly in this region, but could account for a large part of absorbed light in the chlorophyll *b*-deficient red algae.

This doublet structure, anticipated by Förster (1951, 1959), was found by Lavorel (1957) in the spectrum of dimeric molecules of several pigments; Brody (1958) suggested that dimetric chlorophyll molecules are the cause of the "red drop" in the action spectrum of photosynthesis. Krasnovsky *et al.* (1955, 1957) had postulated even earlier that the "inactive" part of chlorophyll *a* is in an "aggregated" form.

Brody suggested that a greater relative concentration of the dimeric form in red algae can explain quantitatively the difference between the action spectra of the green and the red algae: but the hypothesis of a more successful competition of the "inactive" with the "active" form for light energy supply in the 650–680 mμ region, due to the absence of chlorophyll *b*, may provide a better explanation (since the absorption curves in Fig. 8 show no evidence of a significant difference in the concentration ratio of the two components).

The hypothesis of a monomer–dimer equilibrium being responsible for the complex structure of the chlorophyll *a* band *in vivo*, and for the occurrence of the red drop, could be either an alternative or a complement to Franck's hypothesis of a "hydrophobic" and a "hydrophilic" chlorophyll complex.

The nearest interpretation of the "red drop" and the Emerson effect is that the two forms of chlorophyll *a* bring about two different primary photochemical processes, which need to proceed at a balanced (perhaps equal) rate in order to achieve photosynthesis.

Speculations about the nature of these two processes (photoxidation of water vs. photoreduction of CO_2 ? photoreduction of carboxyl groups vs. photoreduction of carbonyl groups ? photoreduction of pyridine nucleotides vs. production of high energy phosphate ?—to mention three obvious possibilities) should be delayed until some experimental evidence becomes available.

12. INHIBITION OF PHOTOSYNTHESIS BY EXTREME RED LIGHT

In the course of the above-described studies, Govindjee *et al.* (1960) discovered an additional effect, which is now being investigated. This is the *inhibition* of photosynthesis, sensitized by light absorption in the region of the red drop, by extreme red light, in the region of 730–770 mμ. This phenomenon is reminiscent (and may or may not be related) to the inhibition of certain morphogenic processes in plants by light in the same spectral region. The latter was found by Butler *et al.* (1959, 1960) to be due to a photolabile pigment dubbed "phytochrome", which can be transformed by illumination with 730 mμ light into a form absorbing at 660 mμ; and by 660 mμ light back into the form absorbing at 730 mμ. Whether the inhibition of photosynthesis by extreme red light, too, is

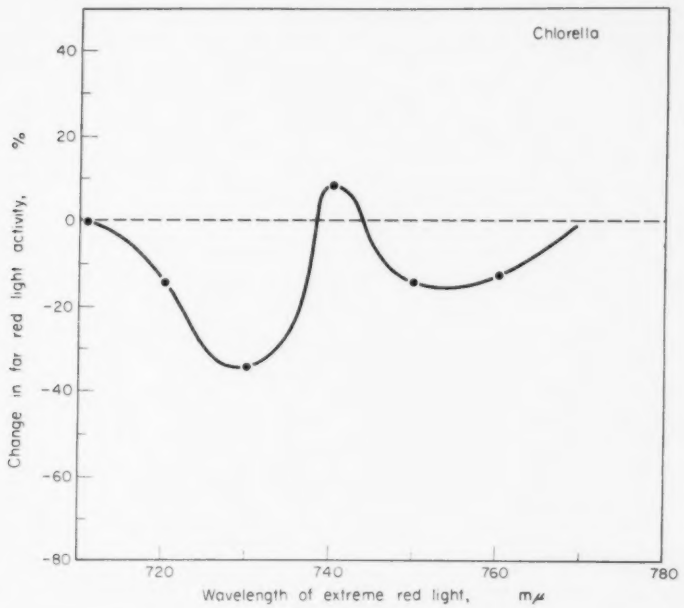

FIG. 13(a).

FIG. 13. Action spectra of extreme red light inhibition of photosynthesis sensitized by far red light. Note two bands, at 730 and 760 mμ. Govindjee *et al.* (a) Photosynthesis in *Chlorella*; (b) photosynthesis in *Porphyridium*; (c) quinone reduction in *Chlorella*; (d) Hill reaction in chloroplasts.

due to the same or similar photolabile pigment needs further study, and the whole matter is better reserved for a later occasion. One point worth emphasizing here is that the inhibition appears to affect only photosynthesis produced by far red light (i.e. in *Chlorella*, light in the region of 680–720 mμ); it does *not* affect photosynthesis produced by light of 650–680 mμ. The inhibition reappears, however, at certain other wave lengths farther towards the blue, so that two characteristic action spectra can be plotted for the whole phenomenon. The first (Fig. 13)

refers to the pigment which causes inhibition; the doublet shape of the band in this spectrum has been confirmed by repeated measurements, and was found also in the study of the Hill reaction (quinone reduction) in *Chlorella* and in pokeweed

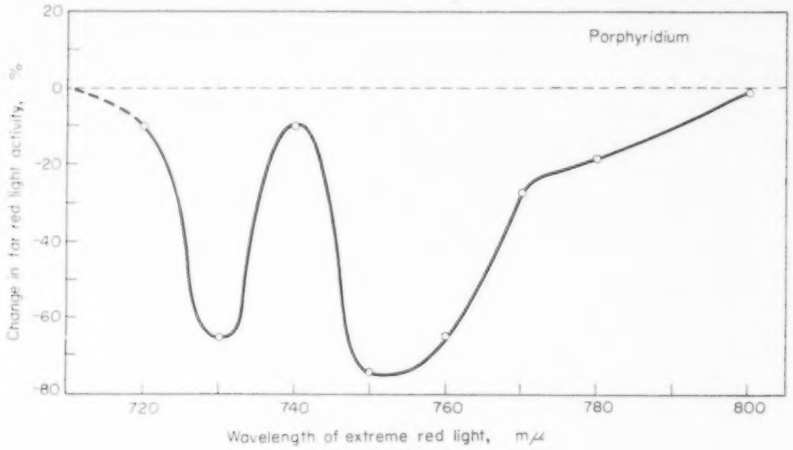

FIG. 13(b).

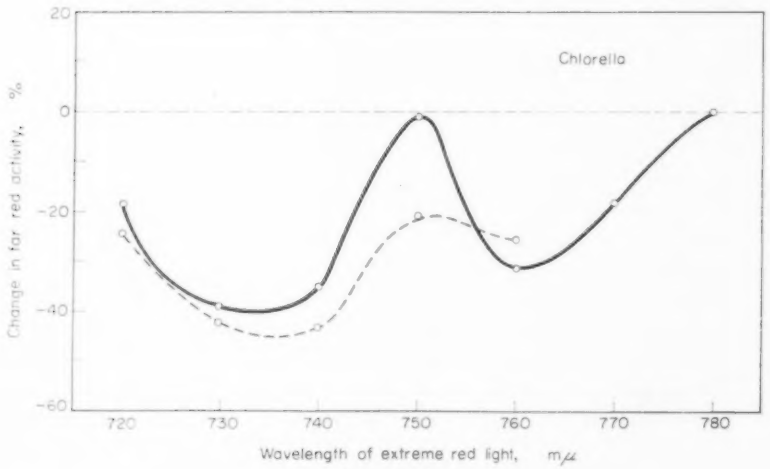

FIG. 13(c).

chloroplasts by Govindjee (unpublished). The other spectrum (Fig. 14) refers to the pigment whose contribution to photosynthesis is inhibited by extreme red light. It should perhaps be called "reaction" rather than "action" spectrum, and should correspond to the absorption spectrum of an "extreme red sensitive" chlorophyll *a* component. It was mentioned above that the chlorophyll component responsible for the red drop should have minor bands in the orange and yellow,

and a "Soret band" in the blue-violet part of the spectrum. A deficiency in the quantum yield of photosynthesis and enhancement of this yield by light absorption in other components of the pigment systems should be therefore observable not

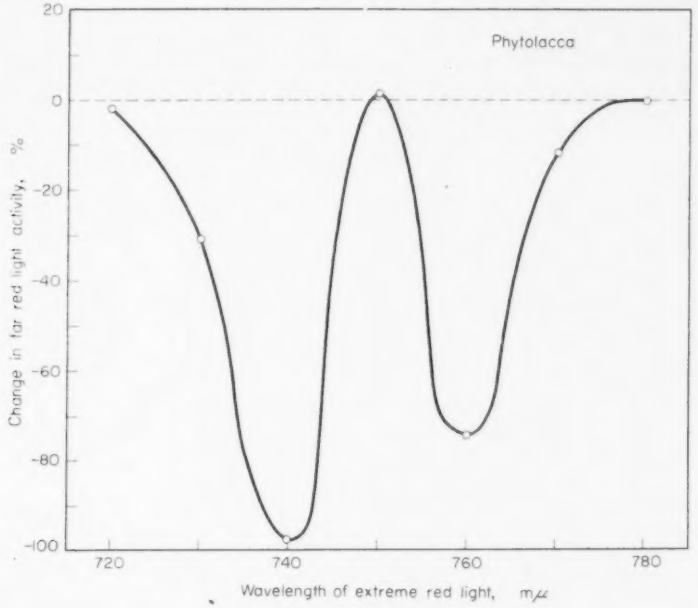

FIG. 13(d).

only > 680 mμ, but also in several other regions of the spectrum. It seems that the action spectrum of the inhibition by extreme red light has given us the first experimental evidence in this respect, revealing the spectral bands in which the "inactive" component (Chl 680–690) provides the most significant contribution to the total chlorophyll absorption.

Whether the pigment whose excitation produces the inhibition effect (and which must have absorption bands at 730 and 750 mμ) plays an intrinsic role in the photosynthetic mechanism, we do not yet know. What can be said, however, is that the experiments described in sections 8–12 of this paper prove that the primary process in photosynthesis, and the function of chlorophyll in it, are much more complicated—and correspondingly more challenging—than had been thought only a year or two ago.

This paper summarizes work which my co-workers Govindjee, Rajni Govindjee and Carl Cederstrand have carried out in the last two years. To them, and to J. B. Thomas (Utrecht), in 1959–60 visiting research professor at the University of Illinois, I would like to express my recognition for the work carried out largely on their own initiative and responsibility. The technique of the studies—combination of precise manometry and spectrophotometry—was developed in long and patient

work by the late Professor Robert Emerson; and the work described here was the logical development of Emerson's own work on the "red drop"—a study which had occupied his attention for several years before his death in an aeroplane crash in February 1959. The research described here owes much to his original inspiration.

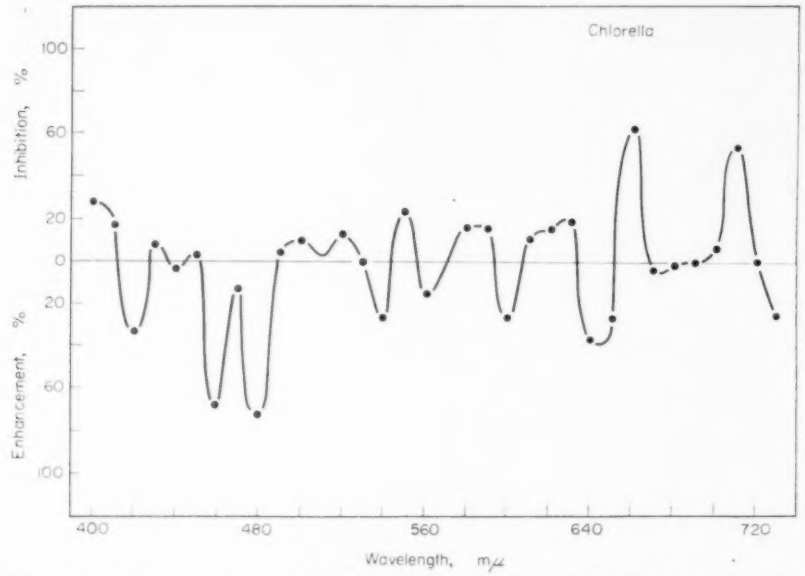

FIG. 14. Action spectrum of sensitivity to extreme red inhibition. Govindjee (in preparation).

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THE WAY OF CO_2 IN PLANT PHOTOSYNTHESIS*

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INTRODUCTION

THE basic biochemical pathways through which carbon dioxide is reduced during photosynthesis in algae have been mapped (Bassham *et al.*, 1954; Calvin, 1956; Bassham & Calvin, 1957). A principal feature of these pathways is the carbon reduction cycle. A simplified version of this cycle is shown in Fig. 1. In order to study these paths, Calvin and co-workers gave radioactive compounds, such as $^{14}CO_2$ and $KH_2\,^{32}PO_4$, to photosynthesizing plants (Bassham & Calvin, 1957; Benson, 1951a, b; Benson *et al.*, 1950, 1952a; Calvin & Benson, 1949; Goodman *et al.*, 1953, 1955). The plants made various reduced organic compounds from these labeled substrates. The photosynthesizing plants were then killed by reagents which denatured their enzymes. The soluble compounds were extracted from the dead plant material. This extract was analyzed by two-dimensional paper chromatography and afterwards the radioactive compounds were located on the chromatograms by radioautography. The compounds were identified and their radioactive content determined. From the amount and location of radioactive elements within compounds following exposures of the plants for various lengths of time and under various environmental conditions, the sequence of biochemical intermediates was found.

In the studies reported here we have extended our information about these pathways by more precise control of the environmental conditions during exposure of the plants to tracers. At the same time we have made measurements of the rate of entry of tracer into the plant and of the rate of appearance of the tracer in specific compounds.

We sought answers to the following questions:

(1) How much of the total carbon taken up by the plants enters the metabolic network via carboxylation of ribulose diphosphate (reaction 1)?

(2) How much of the total carbon taken up enters by carboxylation of phosphoenolpyruvic acid (PEPA) (reaction 6)?

(3) Are any other carboxylation reactions, such as the carboxylation of γ-aminobutyric acid (Warburg, 1958), of any importance in steady state photosynthesis?

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† Research Professor of Chemistry in the Miller Institute for Basic Research in Sciences, 1960–61.

(4) Does the carboxylation of ribulose diphosphate *in vivo* lead to one product only (PGA) or does it lead to two products (PGA and some other three-carbon compound)?

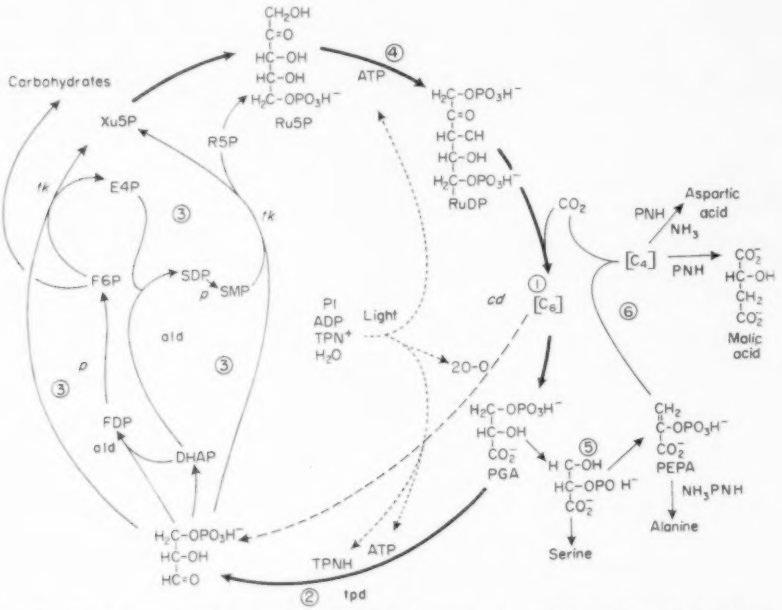

FIG. 1. Carbon reduction cycle (simplified version). (1) Ribulose diphosphate reacts with CO_2 to give an unstable six-carbon compound which splits to give two three-carbon compounds. At least one of these is 3-phosphoglyceric acid. The other three-carbon compounds might be either 3-PGA, as it is known to be in the isolated enzyme system, or some other three-carbon compound such as a triose phosphate (dashed arrow). (2) PGA is reduced to triose phosphate with ATP and TPNH derived from the light reaction and water. (3) Various condensations and rearrangements convert the triose phosphates to pentose phosphates. (4) Pentose phosphate is phosphorylated with ATP to give ribulose diphosphate. Further carbon reduction occurs via conversion of PGA to phosphoenolpyruvic acid (5) and carboxylation (6) to form a four-carbon compound (probably oxaloacetic acid). Reactions leading to the formation of some of the secondary intermediates in carbon reduction are shown.

(5) What are the rates of flow of carbon through amino acids during photosynthesis and what portion of the total carbon uptake can be ascribed to amino acid synthesis?

(6) Is there more than one reservoir of each amino acid? If so, what are the concentrations of the reservoirs directly involved in photosynthesis?

(7) What are the metabolic pathways leading from primary products of photosynthesis to these amino acids, and are any of these amino acids themselves primary products of carbon reduction?

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(8) What is the explanation of light-dark transient effects on the rate of synthesis of various amino acids ?

EXPERIMENTAL

The plants used in all experiments were the unicellular green algae, *Chlorella pyrenoidosa*, raised in continuous automatic culture tubes as described previously (Bassham & Calvin, 1957). The algae were raised and harvested as a 0·5 per cent

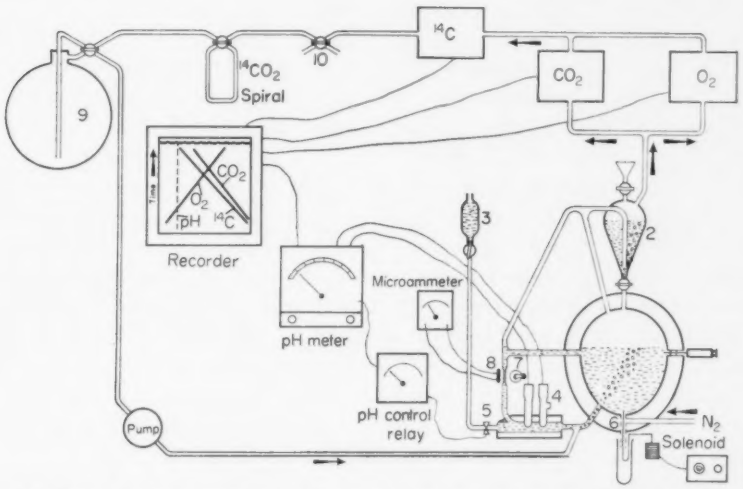

FIG. 2. Steady state apparatus. (1) algae chamber, (2) water or nutrient solution reservoir, (3) acid or base reservoir, (4) pH electrodes, (5) solenoid operated pH control valve, (6) solenoid operated sampling valve, (7) small lamp, (8) photovoltaic cell, (9) large gas reservoir, (10) four-way stopcock.

(volume wet packed cells/volume) suspension. The algae were centrifuged from the culture medium and then suspended in a special nutrient solution. This suspension (80 ml) was placed in the illumination chamber of the steady state apparatus. This apparatus and its use for steady state experiments has been described in detail elsewhere (Bassham & Kirk, 1960; Smith *et al.*, in press). Briefly, it consists of an illuminated vessel to contain the suspension of photosynthesizing algae, and a closed system for circulating gas. This gas passes through the suspension and through instruments which measure the levels of O_2, CO_2 and $^{14}CO_2$. The pH and density of the algal suspension are maintained constant automatically during the course of the experiment. The signals from the measuring instruments are continuously recorded, giving a record of the changes in gas tensions due to photosynthesis. Together with the physical constants of the system (volume, sensitivity, etc.) this record permits calculation of the photosynthetic rates of CO_2 and ^{14}C uptake and O_2 evolution. The apparatus is shown in simplified, schematic form in Fig. 2.

The voltage of the lights illuminating the algae is usually adjusted to just give light saturation at 25°C and with 2% CO_2 in air bubbling rapidly through the algae. The algae are allowed to photosynthesize for about an hour with ordinary CO_2 before admission of ^{14}C. Then $^{14}CO_2$ is added to the gas phase (by turning a stopcock) and H $^{14}CO_3^-$ solution is injected into the algae suspension simultaneously. The specific radioactivities of these two chemicals are so adjusted as to bring the specific radioactivity of both the gas and liquid phases in the closed system immediately to their final levels (Bassham & Kirk, 1960).

After addition of $^{14}CO_2$ to the photosynthesizing algae, samples are rapidly taken by means of the solenoid operated sampling valve (Fig. 2). Plant material in each sample is killed by mixing it with methanol to a final concentration of 80% methanol (by volume). Later, samples are taken less frequently, until the active reservoir of early intermediates in the carbon fixation pathways are saturated with ^{14}C at the same radioactivity as the administered $^{14}CO_2$. Sometimes the experiment is then stopped. In other cases, some environmental factor is changed (e.g. the light may be turned off) and samples are again taken to follow the change in the labeling pattern which results from the changed conditions (Bassham & Kirk, 1960; Smith *et al.*, in press). Several such experiments will be described later.

The killed samples are weighed, extracted with 80% methanol in water, 20% methanol in water, and water, and the extracts are combined. The combined clear extracts are concentrated at reduced pressure at below room temperature. The concentrated extract or an aliquot portion thereof is transferred quantitatively to the paper chromatogram and analyzed in two dimensions (phenol–water, butanol–propionic acid–water) as in earlier work (Benson *et al.*, 1950). The location of the radioactive compounds on the chromatogram is found by radioautography with X-ray film. When necessary, overlapping phosphate esters are eluted, treated with phosphatase and rechromatographed (Bassham & Kirk, 1960).

Determination of radioactivity in compounds

The amount of radiocarbon in each compound of interest on the chromatograms from each sample is measured with a Geiger–Mueller tube (Bassham & Kirk, 1960).

Measurements of the rates of CO_2 uptake, ^{14}C uptake and O_2 evolution by the photosynthesizing algae are made by taking the slopes of the three traces on the recorder (Bassham & Kirk, 1960). From the $^{12}CO_2$ reading and the ^{14}C reading the specific radioactivity of the CO_2 may be calculated at all times during the experiment. This specific radioactivity is used to convert the rates of change of radioactivity in the system to rates of change of what we shall call "^{14}C" throughout this paper. For convenience of expression and calculation, this ^{14}C will be expressed in μmole and represents the amount of ^{12}C and ^{14}C corresponding to a given measured amount of radioactivity in the CO_2 administered to the algae at any time during the experiment.

In some experiments, small aliquot portions of each sample of algal material, taken and killed in alcohol during the course of the experiment, are spread in a thin

layer on planchets with acetic acid, dried and counted. The amount of ^{14}C found at each time of exposure of the algae to $^{14}CO_2$ is plotted and the slope of the curve drawn through these points gives the rate of appearance of ^{14}C in stable compounds in the plant.

After the ^{14}C in individual compounds found on the paper chromatogram has been measured, the amounts are sometimes totaled for each sample up to 1 min, and a rate of appearance of ^{14}C in these compounds is calculated.

The concentration of carbon in a reservoir of a biochemical intermediate compound which is rapidly formed and used may be measured. This is accomplished by allowing the molecules of this compound to reach a constant level of labeling with ^{14}C. At this time the specific radioactivity of this pool of the given compound is the same as that of the carbon dioxide which is administered to the plant. Such a pool will be referred to as the "active" pool. We desired to measure the concentration of compounds in the "active" pools. As mentioned before, we shall express as μmole of ^{14}C the amount of ^{12}C and ^{14}C in μmole which corresponds to the measured amount of radioactivity, using the specific radioactivity of the administered $(^{14+12})CO_2$. In other words, if S = specific radioactivity of the CO_2 (in $\mu c/\mu$mole) and A = radioactivity (in μc) found in a given compound from 1 cm^3 of wet packed algae, then A/S = μmole of "^{14}C" in 1 cm^3 of algae.

When the radioactivity of a given metabolic pool of a compound no longer increases as a function of the time of exposure of the photosynthesizing algae to $^{14}CO_2$, the pool is said to be "saturated" with ^{14}C, and the μmole of "^{14}C" as defined above is considered to be equal to the steady state concentration of the metabolic pool. A given metabolic pool is usually only one of several pools of the same compound in the plant. Therefore the total cellular concentration of a given compound is usually greater than the measured "active" pool.

In the case of the sugar phosphates of the carbon reduction cycle, and of 3-phosphoglyceric acid (PGA), the active pools saturated in 3–5 min after the introduction of $^{14}CO_2$, and there is seldom any problem in deciding what the active pool concentration is (Bassham & Kirk, 1960; Bassham *et al.*, 1956). Some amino acids saturate quickly also and thus their levels can be easily determined (Smith *et al.*, in press). In the case of other amino acids, slowness of the active pools to saturate, coupled with appreciable rates of labeling of other pools of the same compound, have made measurement of the active pool size difficult or at times impossible.

In order to provide a comparison between active pool sizes and total concentrations, a method was developed to estimate the very small amount of amino acids in spots on the chromatograms. In these studies, we had to chromatograph the extract from no more than 5 mg of algae, owing to the necessarily high salt content of the extracts. The amino acid was eluted from the paper chromatograms into a small volume. The eluate was made alkaline, swept with NH_3-free air and then buffered at pH 5 by addition of citric acid. Ninhydrin solution was added and the mixture heated to develop color. Finally the estimation of the amino acid was made by measuring the color developed with a spectrophotometer (Smith *et al.*, in press).

CARBON REDUCTION CYCLE

Our first objective was to compare the externally measured rate of ^{14}C uptake with the appearance of ^{14}C in stable compounds. In a typical experiment, the externally measured rate of uptake of ^{14}C was about 15 μmole/min/cm^3 of wet packed algae, that is, 200 ml of 0·5 per cent algal suspension (Bassham & Kirk, 1960). The radioactivity of nonvolatile stable compounds in each sample was determined. Aliquot portions of the samples were dried on planchets and their radioactivity was counted. When results of these counts were plotted vs. time of sampling, the rate of fixation of ^{14}C into compounds stable to drying on the planchets was found to be about 15 μmole/min (Fig. 3).

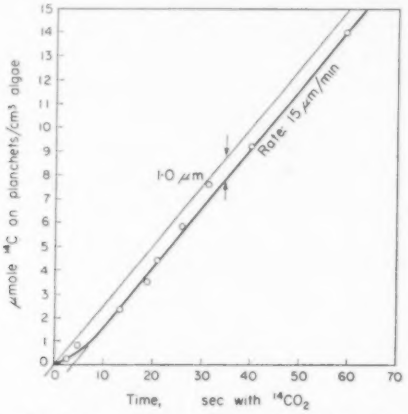

FIG. 3. Appearance of ^{14}C in stable compounds (dried on planchets). *Chlorella pyrenoidosa* vs. time of photosynthesis with $^{14}CO_2$.

After chromatographic separation of the compounds, radioautographs, of which Fig. 4 is typical, were obtained. The radioactivity of each compound in each sample was determined and the total radiocarbon found in the various compounds was plotted against time (Fig. 5). The maximum slope of the curve in Fig. 3 is 13 μmole. This is a lower limit for the rate of appearance of ^{14}C in soluble compounds which are also stable to chromatography. It does not take into account other compounds too weakly radioactive to be counted or "lost" from the front of our chromatograms.

After 30 sec appreciable amounts of radioactivity are passing through the extractable precursor compounds seen on the chromatograms into nonextractable substances which are not seen on the chromatograms. Consequently the rate of appearance of ^{14}C in compounds on the paper decreases.

During the first 10 sec, the rate of appearance of ^{14}C in stable compounds is less than the maximum rate during the subsequent time. This may be due to the presence of an intermediate pool of either HCO_3^- or some other unstable or volatile compound. Such a compound would precede the stable soluble compounds in the fixation pathway. The size of this "pool", if it exists, cannot be greater than the

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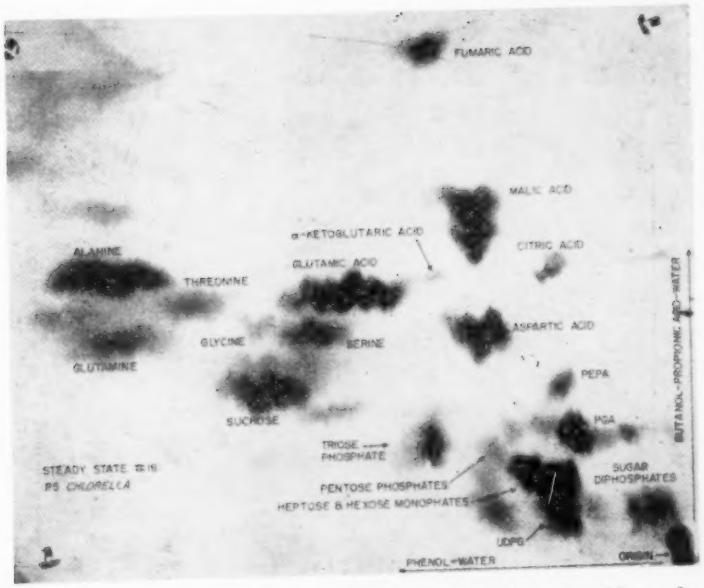

FIG. 4. Radioautograph of chromatogram of *Chlorella pyrenoidosa* after two minutes' photosynthesis with $^{14}CO_2$.

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difference between the fixation curve after 10 sec and a line of the maximum slope drawn through the origin (see Figs. 3 and 5). This is no more than 1·0 to 1·5 μmole, which is equal to the carbon fixed in 4–6 sec in this experiment. The unstable pool may be partly the intracellular CO_2 and partly the unstable carboxylation product which may undergo decarboxylation.

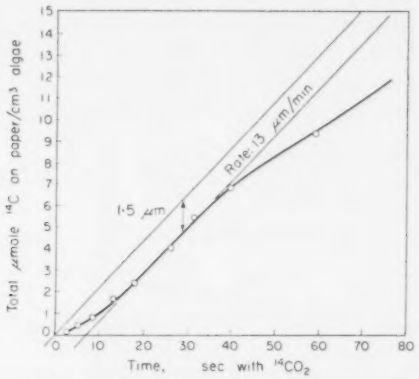


FIG. 5. Appearance of ^{14}C compounds on chromatograms prepared from *Chlorella pyrenoidosa* vs. time of photosynthesis with $^{14}CO_2$.

We may summarize results thus far:

(1) The appearance of ^{14}C in stable, nonvolatile compounds after the first 10 sec of exposure of the plant to $^{14}CO_2$ is equal to the rate of total uptake of $^{14}CO_2$ within experimental error.

(2) During the period between 10 and 30 sec exposure to $^{14}CO_2$, the appearance of ^{14}C in individual compounds which can be isolated by our methods of paper chromatography is equal to at least 85 per cent of the rate of total uptake.

(3) If there is a pool of CO_2, HCO_3^- or other unstable or volatile compound lying between administered CO_2 and stable compounds in the fixation pathway, its amount is not more than 0·5–1·5 μmole (4–6 sec fixation) and it is essentially saturated after 10 sec.

Let us next consider the question of how much of this fixed ^{14}C must pass through the PGA pool. We must first consider whether or not the labeled PGA that we find is a real intermediate in the carbon fixation pathway. Moses & Calvin (1958) found evidence for the formation of an unstable six-carbon product of the carboxylation of ribulose diphosphate which was tentatively identified as 2-carboxy-4-ketopentitol-1,5-diphosphate. This compound appears to be unstable towards decomposition both to CO_2 and ribulose diphosphate and to two molecules of PGA, depending probably upon the conditions to which it is exposed. Doman (1959) demonstrated the presence of an unstable early CO_2 fixation product in leaves. Doman's compound is reported to be labeled prior to PGA, and appears to undergo biochemical conversion to PGA. It is also reported to be chemically converted to PGA under ordinary conditions of analysis.

The accumulation of labeled PGA when the light is turned off following a period of photosynthesis in algae with $^{14}CO_2$ (Calvin & Massini, 1952; Bassham *et al.*, 1956) and the apparent conversion of PGA to alanine, serine and other products (Bassham, 1959) may be considered as evidence for the biochemical role of PGA as an intermediate in the carbon cycle. Park and co-workers (1960) have shown that PGA-1-^{14}C is readily reduced by a sonically ruptured chloroplast system in the light. This reduction is inhibited by threose 2,4-diphosphate. When the same system is allowed to fix $^{14}CO_2$ in the light in the presence of threose 2,4-diphosphate, labeled PGA accumulates. It may be concluded from this that PGA is a biochemical intermediate in the major pathway of CO_2 reduction during photosynthesis.

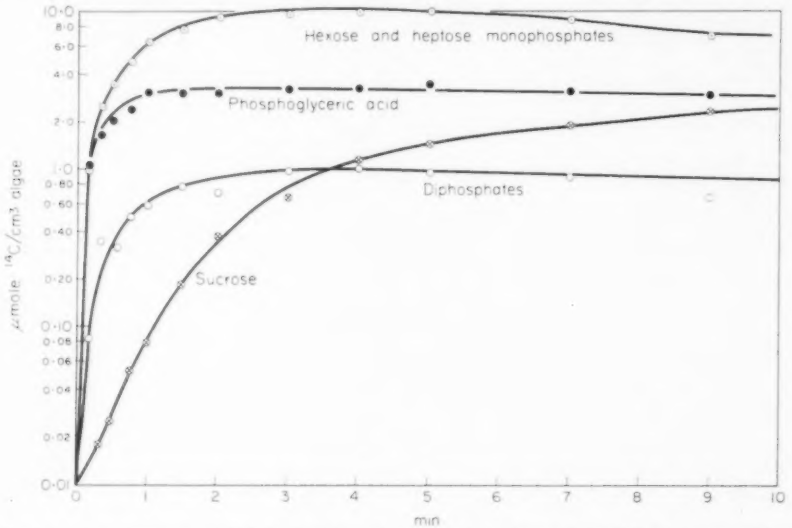


FIG. 6. Appearance of ^{14}C in PGA and sugar phosphates in *Chlorella pyrenoidosa* vs. time of photosynthesis with $^{14}CO_2$.

The fact that some part of the labeled PGA found in our experiments may be the result of chemical breakdown of the unstable six-carbon carboxylation product does not appear to place any serious limitation upon the interpretation of our kinetic data, since the biochemically formed PGA comes from the unstable keto acid in any event. The total PGA found can be considered as a single pool. The only problem arises in connection with the suggestion to be made below, that in living cells the unstable beta keto acid may be converted biochemically to only one molecule of PGA (containing the most recently introduced carbon) and one molecule of triose phosphate (dashed line, Fig. 1). In that event, nonenzymic conversion of keto acid to *two* molecules of PGA decreases somewhat the numerical difference in degree of saturation of PGA and RuDP leading to the conclusion.

In Fig. 6 are shown the labeling curves of some of the more rapidly labeled compounds and groups of compounds. By 3 min compounds of the carbon

reduction cycle are essentially saturated with radiocarbon. Secondary intermediates such as sucrose, malic acid and several amino acids are not saturated until longer times (5–30 min). In order to evaluate the importance of the fixation pathway leading through PGA we tabulated the actual measurements of ^{14}C found in compounds during the first minute (Bassham & Kirk, 1960). The ^{14}C found in all those compounds thought to be derived from PGA without further carboxylation was added together (T_1). Compounds labeled by C_3–C_1 carboxylation were totaled separately (T_2). Since three of the carbon atoms in these latter compounds are derived from PGA, their total radioactivity was multiplied by a factor which is three to four times the degree of saturation of the PGA, which was presumed to be the same as that of their immediate precursor, namely, PEPA. (The saturation curves for PGA and PEPA are, in fact, very similar in this and other experiments.) The sum of T_1 and T_2f, representing measured ^{14}C which we considered to be derived from the primary reaction which forms PGA, is plotted in Fig. 7. Again, the "pool" of HCO_3^- or other volatile or unstable compound is about 1 μmole and

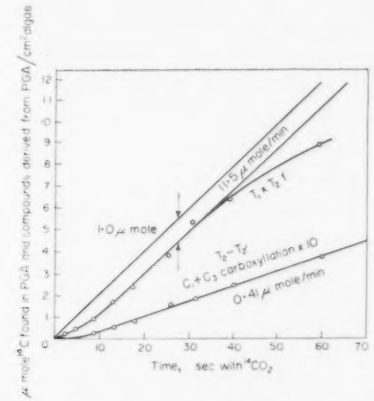

μ mole ^{14}C found in PGA and compounds derived from PGA/cm³ algae
Time, sec with $^{14}CO_2$
11·5 μ mole/min
$T_1 + T_2 f$
1·0 μ mole
$T_2 - T_2 f$
$C_1 + C_3$ carboxylation x 10
0·41 μ mole/min

FIG. 7. Appearance of ^{14}C in compounds derived from PGA and in compounds derived from $C_1 + C_3$ carboxylation in *Chlorella pyrenoidosa* vs. time of photosynthesis with $^{14}CO_2$.

in this case it must precede PGA in the chain of reactions. The maximum rate of appearance of ^{14}C in these compounds falls somewhere between 11 and 13 μmole/min. Thus, on the basis of the appearance of ^{14}C in these extractable, stable compounds alone, at least 70 per cent of all carbon fixed during photosynthesis (measured externally) is incorporated via the carbon reduction cycle. It must be emphasized that this percentage is a lower limit based only on absolute measurements of identified compounds.

A lower limit for the amount of carbon incorporated via C_1 plus C_3 carboxylation is obtained by plotting $T_2 - T_2f$ (Fig. 7). The minimum rate of this incorporation is about 0·4 μmole/min per cm^3 of algae, or about 3 per cent of the total. Note

that this value is for the actual introduction of CO_2 and does not include the carbon derived from PGA (T_2f). The rate of incorporation of ^{14}C into these three compounds thus accounts for about 4×3, or 12 per cent of the total in the experiment. Other experiments indicate that the relative contribution of C_3–C_1 carboxylation varies considerably and tends to be higher (up to three times that reported in this case) when the rate of CO_2 fixation is greater and when amino acid synthesis is more rapid. In addition to the three compounds listed here, other substances may be derived in part from C_1–C_3 carboxylation, such as glutamic acid and citric acid.

While at least 73 per cent of the total rate of fixation of carbon thus appears to be due to the carbon reduction cycle and C_1–C_3 addition, there was no indication of any other significant fixation pathway (Bassham & Kirk, 1960). For example, the maximum rate of labeling of glutamic acid in the above experiment was only about 0·7 μmole/min or 4·5 per cent of all ^{14}C fixed. Since this rate was found between 5 and 20 min it probably represented labeling of all five carbon atoms of glutamic acid, since the precursors would be at least partially labeled after 5 min. The labeling due to carboxylation reaction would be expected to begin during the first 30 sec, if one is to judge by the other known carboxylation reactions which were discussed earlier. Yet, after the first 31·5 sec, the glutamic acid contained only 0·02 μmole of ^{14}C. Moreover, a γ-aminobutyric acid itself would have to be synthesized from CO_2 (by some as yet unknown) if it were a precursor to glutamic acid and would have to be appreciably labeled by the time glutamic acid reaches its maximum labeling rate. Yet we could detect no radiocarbon in γ-aminobutyric acid in this experiment or others of this series even after the algae have been exposed to $^{14}CO_2$ for 10 min. Clearly, little, if any, of the labeled glutamic acid formed in our experiments is made by carboxylation of γ-amino-butyric acid. Rather, it must arise from other intermediate substances such as those formed by the two carboxylation mechanisms already discussed.

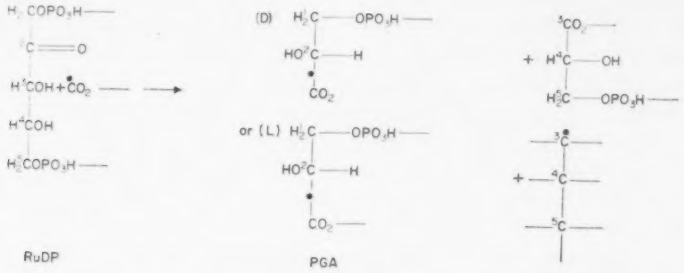
From what we have said above, and from many other studies (Bassham *et al.*, 1954; Benson *et al.*, 1952b) of ^{14}C fixation during photosynthesis, it is clear that the ^{14}C entering the carbon reduction cycle via the ribulose carboxylation passes through the carboxyl group of PGA initially. This is consistent with the fact, established for the isolated enzyme system by Weissbach *et al.* (1956), that the CO_2 is bonded to the No. 2 carbon atom of ribulose diphosphate.

We shall present here an argument, based on kinetic data, which indicates that the carboxylation of RuDP *in vivo* during photosynthesis gives rise to only *one* molecule of 3-PGA, containing the most recently fixed carbon.

If the ^{14}C which has just entered PGA from $^{14}CO_2$ is subtracted from the total ^{14}C in PGA, the ^{14}C in the remaining carbon atoms of the PGA must all be derived from ribulose diphosphate.

The position of the ^{14}C which has just entered the cycle as $^{14}CO_2$ is indicated by the asterisks. In reaction D, there are five remaining carbon atoms of PGA (numbers 1 to 5) which must be derived from RuDP while in reaction L, there are two such "residual" carbon atoms (numbers 1 and 2).

Let us consider the two reactions:



The radioactivity in these "residual carbon atoms" of PGA can be calculated and compared with the radioactivity in the ribulose diphosphate. First the reservoir size of the active pool of PGA was determined. Then the ^{14}C in the carboxyl carbon of PGA was calculated from the rate of flow of carbon through the reservoir. This calculation was made for each of the two cases, reaction L and reaction D (Bassham & Kirk, 1960). Next, this carboxyl group radioactivity was subtracted from the experimentally determined total radioactivity in PGA to give the residual carbon radioactivity at each experimental point. Finally, the radioactivities were divided by the total number of residual carbon atoms, as determined by the pool size and by reaction D or L. This division gives the specific radioactivity, or degree of saturation, at each experimental time. When the specific radioactivities of the residual carbon atoms of PGA, thus calculated according to reactions D and L, were compared with the specific radioactivities of the ribulose diphosphate pool, it was found that reaction D was kinetically not in agreement with the experimental result (Bassham & Kirk, 1960). The residual carbon atoms of the PGA according to reaction D became labeled with ^{14}C more rapidly than the average of the carbon atoms of ribulose diphosphate. On the other hand, reaction L, in which the splitting of the unstable six-carbon carboxylation product results in the formation of only one molecule of PGA (containing the newly incorporated carbon atom), is consistent with the experimental result.

This kinetic interpretation indicates only that the other three-carbon fragment resulting from the splitting off of PGA from the carboxylation product is not a part of the measured PGA "pool". We suggest that the most likely identity of the other three-carbon fragment may be triose phosphate. In that case the split of the carboxylation product would be a reductive reaction. This reaction would thus require TPNH or some other reduced cofactor derived from the light. Whether or not it would also require ATP we cannot say, since the reaction would appear to be barely energetically possible without ATP. This reductive split pathway is indicated by the dashed line in Fig. 1.

AMINO ACIDS

In the early studies of photosynthesis in algae with $^{14}CO_2$, alanine, aspartic acid and several other amino acids were identified as being among the first compounds

to become labeled (Stepka *et al.*, 1948). These compounds, as well as malic acid, were slowly labeled even in the dark when the algae were exposed to $^{14}CO_2$. They were labeled much more rapidly if the algae were photosynthesizing until the moment of addition of radiocarbon. It was recognized that these compounds were products of photosynthetic reduction of $^{14}CO_2$, even though they could also be slowly labeled by reversible decarboxylation reactions of respiration.

Accelerated incorporation of $^{14}CO_2$ into the amino acids of higher plants during photosynthesis has been noted in this laboratory (Aronoff *et al.*, 1947; Benson *et al.*, 1949) and in many others (Nichiporovich, 1955; Andreyeva, 1951; Voskrenskaya, 1953; Nezgovorova, 1959).

Nichiporovich (1955) has presented and reviewed evidence that the synthesis of proteins in the chloroplasts of higher plants is greatly accelerated during photosynthesis. This accelerated protein synthesis appears to utilize intermediates of photosynthetic carbon reduction since the proteins were labeled when $^{14}CO_2$ was administered but not when ^{14}C-labeled carbohydrates were supplied. Sissakian has reported and reviewed experiments that show that protein can be synthesized in isolated chloroplasts from nonprotein nitrogen, including peptides.

TABLE 1—RATES OF CO_2 AND NH_4^+ UPTAKE AND O_2 EVOLUTION OF *Chlorella*

	Time (hr after lights on)	Rate (μmole/min/g wet packed algae)		
		O_2	CO_2	NH_4^+ (upper limit)
Exp. 32	0·5	19·0	19·3	
	1·0			3·0
	2·0			2·6
	2·5	19·6	15·7	2·5
	3·0			2·5
	0–8 min dark			4·3

Holm-Hansen *et al.* (1959) found that the incorporation of $^{14}CO_2$ into amino acids was considerably increased in algae when they were supplied with ammonia. This increase was very marked in the case of glutamic acid. It had previously been found that algae photosynthesizing in distilled water made very little labeled glutamic acid until the light was turned off (Bassham *et al.*, 1956; Calvin & Massini, 1952), whereupon there was a large increase in the labeling of both glutamic acid and citric acid, but no notable increase in labeled alanine.

Our first objective was to follow the labeling of amino acids during steady state photosynthesis and to determine the concentrations of the active pools of these amino acids. These active pool concentrations could then be compared with the total pools of the same amino acids. We also wished to compare the rates of uptake of ^{14}C, CO_2 and NH_4^+ measured externally with the rates of synthesis of these amino acids.

In Table 1 the external rates for a given experiment are given. Fig. 8 shows a typical labeling curve for aspartic acid. From such curves it is possible to calculate both the "active pool" size and the rate of flow of carbon through the biochemical intermediate compound.

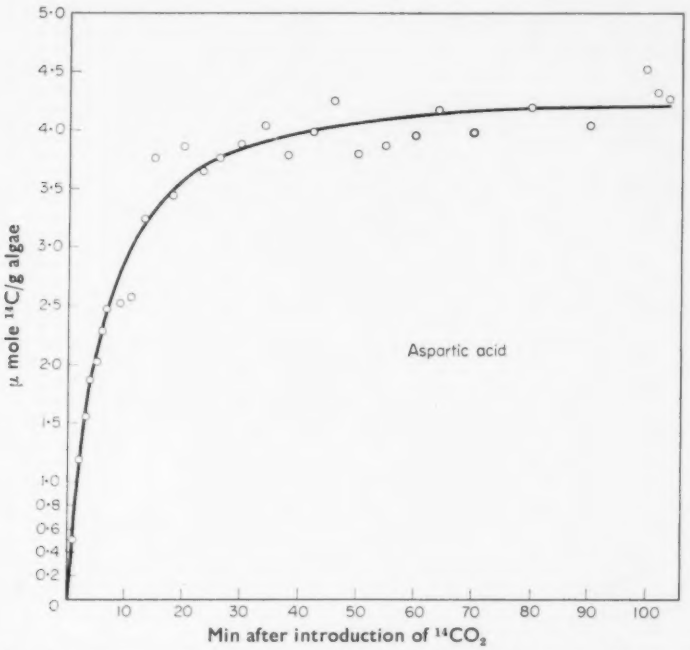


FIG. 8. Appearance of ^{14}C in aspartic acid in steady state photosynthesis experiments.

A comparison of "active" pool concentrations with total concentrations is made in Table 2. Even though there is some uncertainty in active pool sizes of glutamic acid and serine, and considerable variation in total concentration from one experiment to another, it is clear that the rapidly turning over pools, or "active pools", are much less than the total concentrations. The presence of two or more metabolic pools of these amino acids is thus demonstrated.

In Table 3, rates of flow of carbon through some amino acids are given. These rates are based on the steepest part of the labeling curves, corrected for loss of ^{14}C from the pool at the time of measurement (Smith *et al.*, in press).

The results and conclusions regarding amino acids may be summarized as follows:

(1) The "active" pools are only 20–50 per cent of the total pools in the cases of alanine, aspartic acid, glutamic acid and serine. Therefore, two or more pools of each of these amino acids exist in the algal cells.

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TABLE 2—AMINO ACID LEVELS IN STEADY STATE EXPERIMENTS

A summary of amino acid concentrations (expressed in μmole carbon per g wet packed algae) in three steady state experiments. ^{14}C pools measured at time when "active" pools of amino acids become saturated. These values are estimated on the basis of our extrapolation of exponential curves for glutamic acid and serine, levelling off of experimental curve for alanine and aspartic acid.

	Glutamic acid		Alanine		Aspartic acid		Serine	
	μM total carbon	"active" pool μmole ^{14}C	μM total carbon	"active" pool μmole ^{14}C	μM total carbon	"active" pool μmole ^{14}C	μM total carbon	"active" pool μmole ^{14}C
SS 30	47·5	15–20	41·0	15	6·4	3·0	6·3	3·8–4·2
SS 31	43·4	15–20	32·0		7·1	2	6·7	
SS 32	77·5	25–40	42·3	20·0	19·5	4·2	20·2	3·2–4·0

(2) The rates of synthesis of several amino acids from carbon dioxide have been calculated, and are found to account for at least 30 per cent of all carbon fixation during steady state photosynthesis under the chosen environmental conditions. If certain assumptions are correct, the synthesis of these amino acids also accounts for about 60 per cent of all the uptake of ammonium ion, which is the sole source of nitrogen in these experiments.

TABLE 3—RATES OF FLOW OF CARBON THROUGH "ACTIVE" POOLS OF AMINO ACIDS, STEADY STATE EXPERIMENT 32

Compound	Calculated rate of synthesis R μmole of carbon	Equiv. NH_4^+ uptake μmole of NH_4^+
Alanine	2·67	0·89
Serine	0·49	0·16
Aspartic acid	0·89	0·22
Glutamic acid	0·98	0·20
Glutamine	0·32	0·13
Total	5·44	1·60
Externally measured uptake	17·0	2·6
% of total through these pools	32%	61%

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(3) The net increase of ^{14}C in alanine, serine and aspartic acid reaches maximum rates by 5 min after the introduction of $^{14}CO_2$ at the same time that intermediates of the carbon reduction cycle of photosynthesis have become saturated with ^{14}C. This indicates that the immediate precursors of these amino acids are intermediates in the carbon reduction cycle, or are in isotopic equilibrium with intermediates of the cycle (e.g. phosphoenolpyruvic acid) (Smith *et al.*, in press).

In isolated chloroplasts from Swiss chard, Tolbert (1958) has shown that intermediates of the carbon reduction cycle and related compounds (phosphate esters of sugar and carboxylic acids) do not diffuse out of the chloroplast. He found, instead, that ^{14}C-labeled compounds in the supernatant fluid following $^{14}CO_2$ fixation in the chloroplasts were glycolic acid and sucrose, and, to a lesser extent, some free carboxylic acids, and serine and glycine. It is also noteworthy that alanine and aspartic acid became labeled in the chloroplasts but did not diffuse to the supernatant fluid. If we may assume that chloroplasts from *Chlorella* and Swiss chard are similar with respect to formation and retention of these compounds, we are led to the conclusion that:

(4) The rapid synthesis of the amino acids found in the "active" pools in our experiments takes place within the chloroplasts.

(5) Since carbon is not only going into these pools during steady state photosynthesis but is also passing through them; and since the less active pools of the same amino acids (presumed to be outside the chloroplasts) are not becoming labeled at an appreciable rate, it follows that more than 60 per cent of the protein

synthesis in *Chlorella* under these conditions of photosynthesis takes place in the chloroplasts, via free amino acids.

These conclusions find further support in another experiment in which unlabeled acetate was added to algae which had been photosynthesizing in the presence of $^{14}CO_2$. Analysis of ^{14}C and total carbon in glutamic acid showed a rapid synthesis of glutamic acid from unlabeled carbon but no increase in the rate of synthesis from ^{14}C. Thus there appears to be a synthesis of glutamic acid entirely from acetate rather than by a reaction of acetate with some photosynthetic intermediate. This is what one would expect if incremental synthesis of glutamic acid from acetate takes place at a site removed from the site of photosynthesis of glutamic acid (Smith *et al.*, in press).

If, after a period of photosynthesis with $^{14}CO_2$, the light was turned off and the experiment continued, the radioactivity in several amino acids at first decreased and then increased to higher levels than before. At the same time, there was an apparent increase in the rate of ammonia uptake. Various explanations of this effect are possible (Smith *et al.*, in press; Bassham *et al.*, 1956; Calvin & Massini, 1952). We would now favor the hypothesis that when the light is turned off, there is an increased release of radioactive compounds from the photosynthetic site in the chloroplast to the cellular space outside the chloroplast (Smith *et al.*, in press). As a result of this increase in substrate material into the extrachloroplastic space, there would be an accelerated synthesis of amino acids and other compounds in the nonphotosynthetic pools.

When the light is turned off, the formation of ATP and TPNH stops inside the chloroplast. Then synthesis of larger molecules inside the chloroplast no longer occurs. As a result, some of the intermediate compounds are no longer used for photosynthesis.

For example, we think that fatty acid synthesis proceeds inside the chloroplast via acetyl coenzyme A. We think that this acetyl coenzyme A is formed directly from photosynthetic intermediates, such as the sugar phosphates, and free acetate is not an intermediate. However, TPNH from the light is required for the conversion of this acetyl coenzyme A to fatty acids. Thus when the light is turned off, the acetyl coenzyme A, no longer used in synthesis, undergoes hydrolysis to free acetate. This free acetate would more readily diffuse out through the chloroplast membrane into the cytoplasm, where it could then be used for nonphotosynthetic synthesis.

SUMMARY

The general view of the photosynthesis of carbon compounds which emerges from the studies of *Chlorella* photosynthesizing in the presence of $^{14}CO_2$ under steady state conditions may be summarized:

(1) The carbon reduction cycle accounts for most of the carbon dioxide taken up during photosynthesis. Additional amounts of carbon dioxide are fixed by cyclic carboxylation of three-carbon acids, derived from the carbon reduction cycle.

(2) Following the carboxylation step of the cycle, the resulting six-carbon beta keto acid is hydrolytically split *in vitro* to give two molecules of PGA. It appears that *in vivo* only one molecule of PGA containing the most recently incorporated CO_2 is formed. If the splitting reaction is reductive, the other three-carbon compound formed would be triose phosphate.

(3) Several amino acids, particularly alanine, aspartic acid, serine, and glutamic acid, are labeled rapidly with ^{14}C and account for the major part of carbon fixed into amino acids. These amino acids appear to be formed rather directly from intermediates of the carbon reduction cycle, probably in the chloroplast. It appears that rapid protein synthesis occurs in the chloroplast, directly from CO_2 via the carbon reduction cycle and free amino acids. Other pools of the same amino acids exist outside the chloroplasts. Extrachloroplastic synthesis of amino acids can take place during photosynthesis or darkness, using biochemical intermediates released from the chloroplasts or administered to the algal suspension.

(4) The accelerated synthesis of glutamic acid, observed on turning off the light, is attributed to release of biochemical intermediates from the chloroplast to the cytoplasm.

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EVOLUTION OF PHOTOSYNTHESIS

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PRIMARY PHOTOCHEMISTRY ON EARTH

FOR the purpose of this essay I shall assume that the Proceedings of the First International Symposium on the Origin of Life on the Earth and recent reviews relating to our topic (see References) are generally available and therefore may serve as sources for detailed references. Without further discussion of these points where minor disagreements still exist let us presuppose the following as given.

We have had on earth a succession of conditions during which the atmosphere changed from a generally reducing to a strongly oxidizing one. This change of environment was brought about by a rapid loss of hydrogen and a gradual accumulation of oxygen until the present *quasi* steady state was reached. The main source of free energy on the surface of the earth has been and still is, of course, solar radiation. This was (and is) occasionally supplemented here and there by localized volcanic action, which produces heat and reactive chemicals. The change from a reducing to an oxidizing atmosphere was accompanied by an attenuation of the solar spectrum in the ultraviolet region, to the effect that whatever part photochemistry played in the successive creation and accumulation of organic substances and later of living organisms, had to rely in the course of time on the utilization of smaller and smaller light quanta, diminishing in energy from 9 to 1·3 eV/mole but absorbed in increasing amounts by living matter. This decrease in available energy per absorbed light quantum means that the direct photochemical dissociation of water molecules into radicals, as it happened in the beginning, gave way to a very complicated mechanism which in our time achieves the same result with a succession of quanta absorbed by the green pigment chlorophyll.

Furthermore, the photodissociation of the constituents of the earliest atmosphere such as H_2, H_2O, NH_3, CH_4, H_2S, HCN, (O, CO, CO_2) and the subsequent chance recombinations lead to the accumulation of large amounts of aliphatic acid (e.g. CH_2COOH), amino acids (e.g. CH_2NH_2COOH) and of small amounts of oxygen and the oxides of carbon. By the simple means of thermal dehydration, condensation, dehydrogenation, decarboxylation and photochemical interconversion that were often catalysed by metallic or oxidic surfaces or metal ions in solution, the earth's surface became (perhaps only in selected spots) enriched with a host of substances which were very likely the precursors to the substances now existing as parts of living cells. During the last decade we have become convinced that dicarboxylic acids, urea, purines, carotenes, porphyrins, sterols and

perhaps many more such "biochemicals" can arise in a sequence of spontaneous reactions. We are here mainly interested in the first appearance of colored organic substances which broadened the spectrum of possible photochemical activities. For example, we have learned that the biosynthesis of porphyrin out of acetic acid and glycin proceeds over intermediate stages that can also happen spontaneously. To a part this has been proven experimentally. The important, often-told and well-known story needs no repetition.

The appearance of colored, fluorescent substances like the porphyrins must have accelerated the interconversion of organic substances tremendously. We shall concentrate our attention on the porphyrins because these molecules are among the few that apparently have exerted their catalytic powers throughout the eons that it took life to develop. This does not mean that other substances absorbing visible light could not have played important roles. The photodissociation of acetone, $CH_2 \cdot CO \cdot CH_3$ at low temperatures by ultraviolet, for instance, leads to the formation of diacetyl, $CH^3 \cdot CO \cdot CO \cdot CH^3$. The latter, a yellow substance, is activated in blue light and has been shown to sensitize further photochemical synthesis; but, on the other hand, diacetyl decomposes quite easily. The porphyrin ring, once formed, has, however, enormous stability in comparison with other active pigments. This may be the reason why porphyrin metal complexes have become the most widely distributed biocatalysts on earth.

With this background we can address ourselves to the question of the evolution of photosynthesis, defined as the photochemical assimilation and transformation of carbon dioxide carbon into carbohydrate and other constituents of living cells.

The task is to find the path that leads smoothly from the simpler reactions which illuminated pure chlorophyll displays in the test tube to the achievement of the living cell. Fifty years of experimentation with model reactions have enabled us to describe with satisfying consistency how chlorophyll reacts in homogenous solution after it has absorbed a quantum of visible light. Many kinds of compounds of importance to the living cell have been so tested. A few such reactions may be mentioned. In presence of oxygen it sensitizes the photoxidation of amines, purines, tyrosin, sulfhydryl groups and ascorbic acid with great efficiency. In absence of oxygen it may catalyse the transfer of hydrogen from a hydrogen donor to an acceptor (from ascorbic acid to pyridine nucleotide). As the only solute in organic solvents it is usually bleached in the light. Such anaerobic reactions are often reversed in the dark. Without causing a permanent chemical change the excited chlorophyll may also induce the isomerization from a *cis* into a *trans* configuration of an unsaturated substance like carotene.

As much as these reactions have been studied and satisfactorily explained, any one or any combination of these are insufficient to account for the role of chlorophyll in the plant. For one thing the reactions observed *in vitro* are not specific for chlorophyll *a* only but are shared by nearly all porphyrins and their derivatives. Yet we cannot ignore the fact that no matter what other substances may be found as constituents of the plastids, photosynthetic reactions occur only in presence of chlorophyll *a*, or the bacteriochlorophyll of the photosynthetic bacteria. And

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chlorophyll *a* is the same in the entire plant kingdom. This exclusiveness goes hand in hand with the prime achievement of the photosynthetic reaction—the release of molecular oxygen from water. It seems that during the transition from chemical evolution to the first organized cell this particular reaction, which requires the interlinked and interdependent co-operation of several light absorption acts, came into existence. So far no one has succeeded in imitating it in a model reaction, nor do we have a generally accepted theory. Under these circumstances the best approach to the problem is to start at the top with the fully developed mechanism of photosynthesis in green plants and to see whether, by analysing its parts, we find sufficient reasons to believe that there might be a correlation between partial functions and their appearance in the course of time.

THE COMPLEXITY OF THE PHOTOSYNTHETIC MECHANISM

Today, in 1961, we are certain that photosynthesis consists of a number of partially autonomous enzymatic reaction systems which cluster around a central pigment complex of high molecular weight having a definite and essential structure. Within this pigment complex happens the conversion of light energy into chemical energy. The result of the light reaction is a pair of reactive chemical species. The nature of these instable "primary products" is unknown and therefore the main subject of current speculation. The complete "primary process" consists of a sequence of two absorption acts which leads to the separation of H from OH in HOH. The achievement of this light reaction consists in the use of water as hydrogen donor for the production of a reducing agent capable of reducing triphosphopyridine nucleotide (TPN) very efficiently. The oxidized part of water, the equivalent to an hydroxyl-radical, is left as a waste product to be disposed of. The present proposals to explain the mechanism of the "photolysis of water" are not as different as the protagonists of various hypotheses try to make them appear. Suffice it to remark here that the pigmented mechanism which makes such an efficient use of absorbed visible light is in itself so complex that it must have had an evolution of its own. Unfortunately there are no clues as to how or when that happened, except that it probably was complete even before the first cell was formed.

In contrast to our ignorance about the primary process we have a reasonably complete understanding of the various reactions following it.

The parts of the photosynthetic mechanisms which can either be observed independently of any photochemistry or at least understood on the level of comparative biochemistry are: the fixation of carbon dioxide from the air, the reduction of the fixation product, the phosphorylation needed to make the first two steps possible, the elimination of the oxidized primary photoproducts either by spontaneous reduction with a rather wide number of hydrogen donors or by evolution of free oxygen. To these we can add a number of artificially induced reactions.

The photochemical mechanism in algae and bacteria may, therefore, be compared to a motor for general purposes. The motor, the driving force, and the transmission belt remain the same, while different tools can be attached to perform

different tasks as needed. We shall assume that the number of metabolic "attachments" has increased one at a time in the course of evolution; and it is uncertain whether we know them all.

Let us now trace the evolution of photosynthesis backwards in an attempt to see each of the known partial reactions as a consequence of definite changes in environmental conditions. I would like to follow Pirie in keeping a clear separation between the second half of evolution—the Darwinian mechanism based on gene mutation in complete organisms—and the first half—the chemical evolution of organic molecules culminating in the inheritance of randomly acquired properties. Pirie showed for this purpose at the Moscow conference the nice diagram in the shape of an hourglass. The middle narrow region signifies the point in time where, at the end of the chemical evolution, the astonishing interdependence of all cellular reactions was finally accomplished.

The first surprise on our trip down the evolutionary ladder is that during the entire epoch of the Darwinian evolution there was no essential change in the principles governing the photosynthetic mechanism. The most primitive organisms have it complete. This becomes much less surprising when we remember that photosynthesis with the evolution of oxygen had to be complete before Darwinian evolution could progress with the development of respiring multicellular organisms. No multicellular, differentiated organisms are known that are anaerobes. They either never existed or could not survive the turn to strongly aerobic conditions. However, our starting point is not at the very beginning of the Darwinian period because we are dealing with complete cells that are far from primitive eobionts (Pirie's word for pre-cellular self-reproducing entities). We should not forget (as Pringsheim has pointed out) that as far as fundamental life processes are concerned there are no primitive organisms left for us to study.

But if life with oxygen is the last phase in evolution, the preceding one is life without oxygen. This trivial ascertainment permits us to conclude that the mechanism for the release of oxygen is the most recent acquisition of the photosynthetic apparatus. Can we make this plausible? And what is left when we take it away?

The evolution of oxygen from water requires at least three steps. During the study of herbicidal chemicals on the metabolism of algae, in particular *Scenedesmus obliquus*, substances have been found that stop plant growth by preventing the release of oxygen in the light. Among these inhibitors some work so specifically that no other metabolic process of importance is touched. Not only do the poisoned algae continue to respire or ferment as usual, but also their photochemical mechanism remains perfectly normal. The test consists in letting them use hydrogen in conjunction with the assimilation of carbon dioxide. The same effect, a specific inhibition of oxygen release only, can be achieved in certain algae by growing them in absence of the customary manganous ions. Both these observations make it extremely plausible that whatever else had to change to make the release of free oxygen possible—an indispensable part of this last evolutionary step must have been the addition of certain catalysts to an already existing complete

mechanism for the fixation of carbon dioxide, for photophosphorylation, and for photoreduction with hydrogen. In place of the reaction leading to molecular oxygen there has been originally a coupling to hydrogenases or dehydrogenases.

With the elimination of oxygen evolution we have arrived on the level of the anaerobic purple and green bacteria. The idea to put them in this place on the phylogenetic scale has been accepted ever since van Niel first proposed it. Let me remark that as long as an organism which contains only chlorophyll *a* or bacteriochlorophyll (and a minimum number of other pigments) can achieve complete photosynthesis with the evolution of oxygen or the oxidation of various hydrogen donors respectively, it is unnecessary for our purposes to ponder why related plants may have developed additional light-absorbing pigments or specificities for other hydrogen donors. Our attitude would be different if together with other pigments we would have found an altogether new type of photosynthesis. This is not the case.

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We have a certain freedom to interpret comparable data obtained from different forms of photosynthetic micro-organisms in favor of any evolutionary theory because it is not to be expected that the algae and bacteria we study today have remained unchanged, just as they were at the start of the aerobic epoch. After all, a billion years must have left a mark even upon organisms which did not evolve much further. We should not see in the present-day purple bacteria, for instance, the direct ancestors of our present-day green algae. To understand better the way photosynthesis evolved we should give more weight to the traits which these organisms have in common than to deviations which can be understood as due to later developments. It is more important that some green and blue-green algae can still perform partial reactions typical for the purple bacteria than that these reactions cannot any more be found in the majority of higher plants. The following are some known examples of common metabolic processes: absorption or evolution of hydrogen gas during the anaerobic photometabolism via an oxygen-sensitive hydrogenase; fixation of molecular nitrogen; photochemical utilization of acetate and, finally, assimilation of carbon dioxide.

The reduction of carbon dioxide is an effect twice removed from the primary process. First, a carboxylic acid has to be photochemically reduced to an aldehyde which can be transformed into various carbohydrates. Second, at least one among these sugars has to be photochemically phosphorylated to function as an acceptor for carbon dioxide in a spontaneous, exergonic carboxylation in order to re-form the same carboxylic acid that started the cycle.

This means from an evolutionary viewpoint that carbon dioxide reduction could have been perfected only after the ways for storing light energy had first been properly developed. It is of interest in this respect that the purple bacteria, as well as some algae, are not well adapted to the production or utilization of carbohydrates, but prefer to orient their metabolism around aliphatic acids, preferably acetic, one of the primary compounds in chemical evolution. Nevertheless, the purple bacteria fix free carbon dioxide by a mechanism very similar, if not identical, to that of the green algae. Hence we assume that an exergonic carboxylation was added

to an already existing simpler photochemical process by developments within an organism antedating both the present day algae and present day purple bacteria.

The newest support for such a view is the existence of some rare green algae that refuse to touch anything else but acetate and behave like obligate photo-organo-troph bacteria. This brings us to the next step down the evolutionary scale, to organisms that neither evolve oxygen nor assimilate carbon dioxide but use light for the assimilation of simple organic molecules into suitable cell material. There seem to be no organisms left that manage to live purely on this reaction and nothing else. The algae just mentioned, while having remained primitive with respect to the *de novo* synthesis of organic compounds, have undergone two mutations, leading to the formation of green chlorophyll and to the respiratory use of oxygen (evolved by other plants).

The next move downward in complexity is quite considerable, but it is well supported by experiment. If we eliminate from the photochemical system all synthetic reactions involving carbon compounds, which experimentally can be done quite easily, we remove the reason for the existence of an organized cell. We are left with the naked chlorophyll complex. Stripped of the cell and of all enzymes necessary for the synthesis of organic cell constituents, it has yet not lost the power to capture light energy for a number of chemical uses. There is no reason why we should not believe that such a complex evolved ahead of the organized cells and existed for a while independently by itself.

CELL-FREE PHOTOSYNTHETIC PARTICLES

An intact chloroplast has many millions of chlorophyll molecules which cover the lamellae shown in microphotographs. But even small fractions of chloroplasts comprising no more than a few hundred chlorophyll molecules are still able to release oxygen from water when given the opportunity to reduce numerous physiological and unphysiological compounds in the light (Hill reaction). These reactions often go faster when coupled to a typical phosphorylating system that saves part of the light energy in simple polyphosphates or in the form of the best known among the ubiquitous biological phosphate compounds, adenosine triphosphate (ATP). This kind of storing light energy proceeds most efficiently when the substance to be reduced in the Hill reaction is an electron transport catalyst which can be reoxidized on the spot by a coupling with intermediates on the path of photosynthetic oxygen. This is Arnon's cyclic photophosphorylation.

The ability to evolve oxygen and that to fix carbon dioxide can both be easily abolished without damage to the photo-oxido-reduction system. Thus it is understandable that the conversion of light energy into ATP could be shown to be independent of the other two reactions.

The question whether photoactive (or photo*synthetic*, if you wish) pigment complexes of the kind extracted from algae or purple bacteria ever existed for themselves in the pre-cellular stage of evolution we can answer with assurance only after they have been artificially compounded in the laboratory. But if they existed

it is easy to see that they fulfilled very useful tasks in an organic soup containing most of the building blocks for a future cell.

What about simplifying the system further? The phosphorylation mechanism can be uncoupled, or the enzyme destroyed, this does not touch the original oxido-reduction mechanism—it makes it only a little more difficult to demonstrate that it is functioning and to find out what the light is doing. We shift, therefore, to physical methods which permit us to observe the primary process directly—the events in the pigment complex following the absorption of light quanta. The automatic recordings of spectral changes are accumulating fast in many laboratories. In such preparations stripped free to the bone of the metabolic reactions which are characteristic for a living cell, we see interesting oxido-reductions going on, for instance, between chlorophyll *a* and cytochromes. Such light-driven reactions will proceed even at low temperatures where ordinary biochemical reactions stop. Because the reacting pigments are known, the spectral changes look familiar—they are similar to what has been seen in test tube models. But we should not forget that the pigment complex still contains macromolecules—proteins and phosphatides. The observed reactions depend on substances which are very advanced on the scale of the purely chemical evolution; and more than that, the structure of this association determines the nature and efficiency of the primary photochemistry.

Attempts to remove or inactivate the remnants of proteins and enzymes of the original photosynthetic mechanism while retaining some specific photochemistry have so far been without success. Our methods are still too crude. When we try to simplify the photochemical redox system still further at least three basic characteristics disappear together. First: the specificity of chlorophyll *a* for what it does in the natural complex. It now reacts, when illuminated, exactly like other related or even unrelated dyestuffs. Second: the secret of a multi-quantum mechanism geared to work as a unit. All remaining photoreactions are of the known one quantum type. Third: the possibility of using water as a hydrogen donor. Some may argue that this was lost at an earlier stage of our analysis. I shall deal with this argument on another occasion. Be it as it may, too many things have changed at once, and instead of continuing our descent step by step into the past of the living photochemistry while taking note of an understandable evolutionary sequence, we have suddenly fallen through a dark hole, as it were, and landed on familiar grounds, namely the photochemistry of assorted pigments mentioned in the beginning.

This obscure passage from oxido-reductions in natural pigment complexes to related reactions in artificially constructed systems is now the front line of research in photosynthesis. It seems that nowhere else in the field of biology are the chances as good as here to discover and understand the step by step transition from an entirely chemical (and artificial) reaction mechanism to one that has first regulated and later determined the evolution of living things. Some progress has also been made here. We know already that the chlorophyll pigment complex is surprisingly durable in the dried state, and that not all coloured components are equally indispensable as is chlorophyll *a*. β-Carotene is not needed for photosynthesis

except in the presence of oxygen. Under aerobic conditions it protects the cells against photo-oxidation. And yet carotenes are already present in those anaerobic organisms, the purple bacteria, which we like to regard as the nearest relatives of those anaerobic organisms that lived in the time before there was any oxygen.

In retracing the developmental steps of the photochemical mechanism in living organisms we lost the path in that region of the time scale where also the traces of other biological reaction mechanisms (if they can be followed so far back) disappear —at the time of the eobionts, when the macromolecules became the means to remove certain basic reagents from the homogenous solution. The two-dimensional arrangement on proteins or interfaces between hydrophylic and hydrophobic compounds produced effects that in many cases were decidedly more than just an acceleration of reactions known to proceed in the same way, though more slowly, in solution. This seems particularly true in the case of photosynthesis, with its amazing overall efficiency.

QUANTUM NUMBERS AND EFFICIENCY

Some ways to trap electrons or protons set in motion by the absorbed light energy will be more efficient than others. Photosynthesis does not depend on random collisions in solutions where it is next to impossible to separate the primary products so as to prevent a recombination. In the living cell a few selected chemicals permanently surround the light absorbing pigment. The chlorophyll is embedded in a structure composed of protein, phospholipids, carotene, quinone, cytochrome and a varying number of other pigments. Whenever this arrangement is disturbed or broken down to the point where less than 200 chlorophyll molecules remain together nothing is left but exergonic one quantum processes.

No one has seen the accumulation of reactive intermediates belonging to the photochemical phase whenever inhibitors block the formation of stable, already recognized products at either end of the photochemical system. In other words, the energy-rich primary intermediates disappear in msec by back or side reactions at the least disturbance of the energy (or electron–proton) transfer system.

It came as a surprise and has remained a puzzle that quantum number measurements on the various forms of photosynthetic reactions either in green plants, the normal or hydrogen-adapted variety, or in bacteria utilizing hydrogen donors of vastly differing free energy content, or in chloroplast preparations, always gave the same answer. Regardless of the overall free energy changes indicated by the chemistry of the process, two quanta are uniformly needed for the final transfer of one hydrogen or of its equivalent. This means that the stoichiometry of the process has remained unchanged as far back in time as we have been able to follow it.

On the other hand, we can make a case for an evolution of photochemical efficiency in terms of light energy stored as chemical energy. The anaerobic bacteria achieve barely half a per cent efficiency for the reduction of carbon dioxide with free hydrogen and little more when using organic compounds as hydrogen donors. In the green plants the same reduction now coupled to the evolution of oxygen amounts to an efficiency of 35 per cent. One-third of the

absorbed light energy is stored as carbohydrate. This increase in efficiency was gained by an extension of the enzymatic system coupled to the original pigmented apparatus. It is certainly true that complete photosynthesis in the green plants is more complex than that in the bacteria, and that, again, fewer steps and catalysts are needed if these micro-organisms dispense also with the reactions leading to the reduction of carbon dioxide.

BACK-REACTIONS, PHOSPHORYLATION AND RESPIRATION

A light absorption act increases the free energy of a molecule by the amount of energy contained in the absorbed light quantum. In order to obtain chemical work from such an increase in free energy (the excited state) it is necessary to prevent the rapid and complete dissipation of the potential electronic energy into thermal vibrations while the excited molecule reverts to its ground state. This immediate heat loss can be postponed by coupling the return to the ground state with a mechanism suitable to convert the short-lived free electronic energy of the excited state into potential chemical energy, in other words coupling it with the synthesis of one or more chemicals which retain a good part of the energy originally delivered by the absorbed light quantum.

The efficiency with which the energy captured in the first pair of oxidized-reduced photoproducts can be utilized in cellular metabolism depends on the number and nature of further intermediate steps before the original energy level of the entire system has again been reached. The evolution of photosynthesis (the later part which we begin to understand) is therefore the story of ever more prolonged and subdivided oxido-reductions and back reactions.

Figure 1 is a scheme illustrating the hypothesis that the various types of photosynthetic reactions now known came about by an increase of the number of coupled oxido-reductions following the primary process. The argument makes use of the known differences in the pattern of light-induced phosphorylation in bacteria and algae. Since three intermediate steps are the minimum to produce free oxygen the scheme is extended in three stages, numbered 1, 2, 3. Simply for reasons of symmetry, we allot also three steps, a, b, c to the formation of reduced pyridine nucleotide. The increasing complexity of a cyclic phosphorylation is indicated by three levels II, III, IV. In reality any intermediate on the right may be able to connect with any one on the left according to affinities and potentials of the factors available.

The interpretation of the scheme goes as follows:

Step I, the primary process, leads in 10^{-7} sec or less to the photoproducts ·H·Chl· and ·Cyt.·(OH)· with a much longer lifetime. How many unsolved problems are to be considered here we shall not discuss. A back reaction inside the pigment complex means loss of the light energy as heat.

Step II provides the first opportunity to avoid the loss of energy by extending the oxidation or reduction to outside molecules (photoreduction) or by offering a faster back reaction, coupled to phosphorylation. This is meant to correspond to the "shorter" phosphorylation cycle in bacteria, adapted algae and bacterial

chromatophores which is not inhibited by DCMU and similar poisons of the oxygen releasing step.

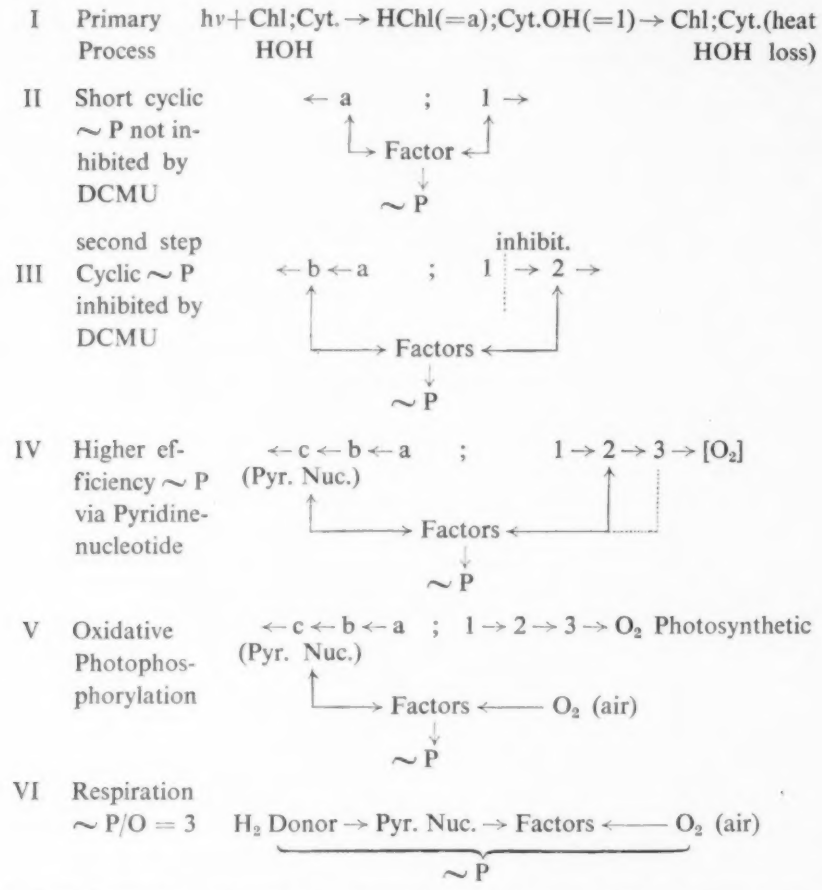
I Primary Process
hν + Chl;Cyt. → HChl(=a);Cyt.OH(=1) → Chl;Cyt.(heat
HOH HOH loss)
II Short cyclic ~ P not inhibited by DCMU
← a ; 1 →
Factor
~ P
second step
III Cyclic ~ P inhibited by DCMU
inhibit.
← b ← a ; 1 → 2 →
Factors
~ P
IV Higher efficiency ~ P via Pyridine-nucleotide
← c ← b ← a ; 1 → 2 → 3 → [O2]
(Pyr. Nuc.)
Factors
~ P
V Oxidative Photophosphorylation
← c ← b ← a ; 1 → 2 → 3 → O2 Photosynthetic
(Pyr. Nuc.)
Factors ← O2 (air)
~ P
VI Respiration ~ P/O = 3
H2 Donor → Pyr. Nuc. → Factors ← O2 (air)
~ P

FIG. 1. Partial evolution of respiration from back reactions in a photochemical system reacting with water.

Step III is different from II because the phosphorylation is now sensitive to DCMU, as found in green plants and chloroplasts.

Step IV has been added because we know that TPN need not be a part of the phosphorylation cycle. When not engaged in storing energy-rich phosphate the reactions II–III promote the various forms of photochemical reduction described in the beginning of this article. Up to here photosynthesis is an *anaerobic* process, because, as has been known since 1953, oxygen is not necessary for any of the

normal photosynthetic reactions also in green plants. Recently Krogman, Vennesland *et al.* discovered that there are phosphorylations in chloroplast preparations that require not only light, but free molecular oxygen. What happens is an exchange of oxygen. The oxygen of photosynthesis escapes while the oxygen of the air is taken up.

The advantage of our scheme is that in it oxidative photophosphorylation finds quite naturally a place as the next evolutionary advance.

Step V is most conveniently explained as a break in the original cycle at points 2 or 3 so that photosynthetic oxygen is evolved, while oxygen of the air becomes the ultimate electron acceptor in the phosphorylation mechanism. This rather odd state of affairs may have once upon a time been of the greatest importance, namely as the transition phase to the appearance of respiration in the living world.

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Step VI is a non-photochemical oxidation system. It comes about when a second break on the reducing side in the mechanism of step V separates the entire phosphorylation chain from the photochemical mechanism. For this to happen all that is needed is a shift of specificities or an exchange of pyridine-nucleotides. This leads to the substitution of independent organic hydrogen donors for those made photochemically. The result is the oxidation of organic compounds by oxygen coupled to the storage of phosphate bond energy, or respiration.

Considering that a complete respiratory mechanism could come into being only after photosynthesis had reached its latest development, and that photophosphorylation is very likely mediated by a chain of catalysts very similar to the oxygen–cytochrome–flavin–pyridine–nucleotide part of many respiratory mechanisms this hypothetical extension of the evolution of photosynthesis seems worth a discussion.

TRANSITION TO AEROBIC CONDITIONS

The transition from anaerobic to aerobic conditions did not conspicuously alter the set of chemicals promoting metabolic reactions. The survival and adaptation to the presence of oxygen required first of all new means to render autoxidations harmless by removing the highly reactive hydrogen peroxide. Some of the iron porphyrins thus became peroxidases and catalases. An important aspect of this is that the very reaction, which was the cause for the accumulation of more and more oxygen, was the one most directly threatened by it. Fluorescent porphyrin derivatives sensitize photoxidation of organic compounds with excellent efficiency at very low oxygen concentrations and iron porphyrins lower the activation energy barrier against spontaneous oxidations by molecular oxygen.

In order to keep functioning, the photochemical part in particular had to be protected against oxygen. Perhaps there have been numerous photosynthetic mechanisms up to this point—and only the chlorophyll *a* mechanism we know survived and flourished actively. The one present in the green and red bacteria survived but could continue its activity only under anaerobic conditions. We have good evidence that the photosynthetic organisms which did survive the shift to oxygen in the atmosphere have apparently been those that happened to contain carotenes in their chromophores; I say happened since the beautiful experiments

of Cohen-Bazire, Stanier and Doudoroff and further new evidence have shown that these carotenes have no ascertained primary function in the photosynthetic mechanism. But in their absence the illuminated chlorophyll complex is extremely sensitive to molecular oxygen and rapidly destroyed by photooxidation.

There seem to be no organisms around that are photosynthetic yet able to grow anaerobically in the dark by means of fermentation. The green plants are unable to grow in the light in the absence of oxygen—that is respiration—even if they may continue to reduce carbon dioxide quite efficiently. Only a few purple bacteria are sitting on the fence—they grow in the light without oxygen—or with it in the dark like the majority of unpigmented bacteria.

The microbes known as strict autotrophs—the photolitho and chemolithotrophs—were once considered the most primitive organisms yet they have to perform the most difficult task of all. So difficult is it indeed to convert carbon dioxide (as the sole carbon source) in contact only with the elements and their inorganic compounds into living matter and to start life in this special way that Haldane and Oparin eliminated the problem by convincing us that it never existed.

But why then do we have a few (very few) such complete chemoautotrophs? The answer is that these must be later evolutionary sidelines that came up where conditions were favorable and that they had no chance for a quick further evolution after one line of complete autotrophs—the photo-lithotrophic green plants—took over the earth.

Our present information on living processes lets us believe that any kind of energy source available on this planet has earlier or later been tentatively put to some use for the differentiation and growth of living things.

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INTERMEDIATE ENZYMATIC FACTORS IN THE COUPLING OF PHOSPHORYLATION TO ELECTRON TRANSPORT*

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The so-called "partial" reactions of oxidative phosphorylation offer an approach to the general nature and sequence of the intermediate steps in energy coupling in the respiratory chain. These reactions include the ATP-ase, ATP–$^{32}P_i$ exchange, the $H_2{}^{18}O$–P_i exchange, the ATP–ADP exchange and the arsenolysis reaction. The enzyme catalyzing the ATP–ADP exchange reaction has been obtained in soluble purified form. It can be recombined with digitonin membrane fragments to reconstruct the dinitrophenol sensitivity which is characteristic of the ATP–ADP exchange in intact mitochondria. Another soluble protein, M-factor, has been obtained which can increase DNP-sensitivity of this exchange. M-factor also increases ATP-ase and ATP–$^{32}P_i$ exchange activity. Under appropriate test conditions both the ATP–ADP exchange enzyme and M-factor preparations can increase the P : O ratio of pretreated digitonin fragments.

Mitochondrial swelling and contraction are dependent on the respiratory chain and its coupling mechanisms. A soluble mitochondrial protein, called C-factor, is necessary to effect contraction of glutathione-swollen mitochondria. This factor has been purified 100-fold. It also exhibits M-factor activity and this finding provides an enzymatic link between the chemical aspects of respiratory chain energy-coupling and the mechanochemical changes involved in mitochondrial contraction. The possible role of the three enzymes in energy coupling is outlined.

THIS paper deals with recent work in this laboratory on (a) the properties of the so-called partial reactions of oxidative phosphorylation and the information they yield on the general nature and sequence of intermediate reactions of oxidative phosphorylation, (b) the separation and properties of three soluble mitochondrial proteins which are involved in these reactions, (c) partial reconstitution of the partial reactions with these soluble factors and (d) the relationship of the molecular factors involved in mitochondrial swelling and contraction of the energy-coupling mechanism. More comprehensive accounts are given in recent reviews (Lehninger, 1960; Lehninger, a, b, in press) and other papers cited.

Current efforts devoted to analysis of the mechanism of oxidative phosphorylation and its *in vitro* reconstruction may be divided into two categories.

The first approach is the direct examination of the ability of specific carriers of the respiratory chain to interact with phosphate or some other energy-conserving molecular component, in the first reaction of the coupling sequence. This approach

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has been taken in some recent studies on the effect of phosphate and ADP on the oxidation state of coenzyme Q (Hatefi, 1959) and on the role of vitamin K (Brodie *et al.*, 1960), the suggestions of Low *et al.* (1958) on the role of flavoprotein in energy conservation, and some recent studies on "complexes" of mitochondrial DPN (Purvis, 1960). None of these studies has yet provided conclusive evidence for either the identity of the coupled carriers or the chemical mechanism of their interaction in the primary energy-conserving step. However, it is obvious that the chemical mechanism of energy conservation at the carrier level is the heart of the problem.

The second approach to the mechanism of oxidative phosphorylation is made possible by the fact that the energy-coupling reactions are reversible, as was first revealed by studies of the so-called "partial reactions" of oxidative phosphorylation, such as ATP-ase activity. Reversibility of the energy-coupling mechanism is given strong supporting evidence by the independent approaches of Chance & Hollunger (1960) and of Azzone, Ernster & Klingenberg (1960). In view of this reversibility it is possible to start from the known end-product of oxidative phosphorylation, namely ATP, and separate and identify the reversible intermediate enzymatic steps involved in the uptake of P_i and ADP, respectively, in the formation of ATP. The latter approach has been studied in this laboratory in some detail.

"Partial" reactions of oxidative phosphorylation. This term designates a series of reactions taking place in mitochondria or mitochondrial fragments which appear to be associated with the coupling reactions of oxidative phosphorylation. These reactions are tabulated in Table 1. The first four reactions have been associated with the mechanism of oxidative phosphorylation because of their

TABLE 1—"PARTIAL" REACTIONS OF OXIDATIVE PHOSPHORYLATION

Reaction	Characteristics	Ref.
1. ATP-ase	Stimulated by DNP	a, b, c
2. ATP–$^{32}P_i$ exchange	Requires ADP; inhibited by DNP	d, e
3. P_i–H_2O ^{18}O exchange	Activated by either electron transfer or ATP; inhibited by DNP	f, g
4. ATP–ADP exchange	Does not require P_i; inhibited by DNP but this inhibition prevented by aging or azide	h, i, j
5. Arsenolysis of ATP	Arsenate competes with P_i; labile arsenylated intermediate probably formed	k, l

(a) Lardy & Wellman (1953); (b) Cooper & Lehninger (1957a); (c) Cooper (1958); (d) Boyer *et al.* (1956); (e) Cooper & Lehninger (1957b); (f) Cohn & Drysdale (1936, 1958); (g) Chan *et al.* (1960); (h) Wadkins & Lehninger (1958); (i) Wadkins (in press); (j) Bronk & Kielly (1958); (k) Crane & Lipmann (1953); (l) Wadkins (1960).

sensitivity to dinitrophenol in uncoupling concentrations: they have been observed not only in intact mitochondria but also in mitochondrial fragments. While dinitrophenol-sensitive reactions are now known to occur in enzyme preparations

which are not capable of phosphorylation (for example, myosin ATP-ase, mitochondrial ATP-ase (Lardy *et al.*, 1953; Cooper, 1958)), the evidence is rather strong that the reactions listed are in fact reflections of the action of the energy-coupling enzymes of the respiratory chain. The DNP-sensitive partial reactions are also inactivated by aging, all at about the same rate, and by U-factor, an endogenous uncoupling agent (Lehninger *et al.*, 1959) which is enzymatically formed at the same rate.

The arsenolysis of ATP discovered recently by Wadkins (1960) provides additional and now rather conclusive proof that arsenate competes with phosphate in the coupling mechanism, which is reflected also by the findings that arsenate uncouples phosphorylation (Crane *et al.*, 1953), and inhibits both the ATP–P_i and the ATP–ADP exchange reactions (Wadkins & Wadkins, 1960). Furthermore, arsenate stimulates respiration in the absence of phosphate or phosphate acceptor as would be expected if a labile arsenylated intermediate underwent spontaneous decomposition. This finding should make possible experimental approaches to the separation of a portion of the coupling mechanism which does not necessarily include the DNP-sensitive site.

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The following reaction sequence summarizes the accumulated evidence from study of the partial reactions (Lehninger, in press):

$$(1)\quad \text{Carrier}_{red} + X \rightleftharpoons \text{Carrier}_{red} - X$$

$$(2)\quad \text{Carrier}_{red} - X \underset{+2e}{\overset{-2e}{\rightleftharpoons}} \text{Carrier}_{ox} \sim X$$

$$(3)\quad \text{Carrier}_{ox} \sim X + P_i \rightleftharpoons \text{Carrier}_{ox} + P \sim X$$

$$(4)\quad P \sim X + E \rightleftharpoons P \sim E + X$$

$$(5)\quad P \sim E + ADP \rightleftharpoons ATP + E$$

The ATP-ase reaction is visualized as reactions 5+4+3, followed by DNP-promoted breakdown of $\text{Carrier}_{ox} \sim X$. The ATP–$^{32}P_i$ exchange is the sum of reactions 3+4+5 and the ATP–ADP exchange as reaction 5. This hypothesis is given additional support by experiments described below.

While it appears probable that these partial reactions are reflections of the action of the coupling mechanism at all three phosphorylation sites in the respiratory chain, there is very little specific information as to whether each site shows these reactions and if so the quantitative contribution each phosphorylation site makes to the measured rate of any one partial reaction in the submitochondrial preparations now available for study. The relative contributions of the three sites can be approximated in the case of the ATP-ase activity; Myers & Slater (1957) have shown that the DNP-stimulated ATP-ases of the three phosphorylation sites differ in optimum pH and in the concentration of DNP required for maximum stimulation. Wadkins (1960) has made the conformatory observation that the arsenolysis of ATP by mitochondria has at least two pH optima. While Low *et al.* (1958) and Grabe (1958) have postulated that the ATP–$^{32}P_i$ exchange is associated only with a single phosphorylation site at the flavoprotein level, our studies of the pH

dependence indicate that all three sites contribute to this exchange. Wadkins has made the important observation that the ATP–ADP exchange reaction of either intact mitochondria or digitonin fragments has only a single pH optimum, but its *inhibition* by DNP or by arsenate has at least two distinct pH optima (Wadkins, in press). This indicates the possibility that the ATP–ADP exchange enzyme may be a single molecular entity, but it must be associated with at least two of the three coupling sites in such a way as to confer arsenate and DNP-sensitivity on this reaction.

It has not yet been possible to date to isolate, in the physical sense, a segment of the respiratory chain containing only a single phosphorylation site for study of the partial reactions at that site, together with their interactions. For this reason, work to the present on partial reactions has dealt largely with three-site systems which make quantitative interpretations somewhat difficult. Nevertheless, nearly all experimental findings indicate that the partial reactions at all sites have quantitatively similar behaviour and these approaches have yielded evidence of significance.

Properties of the ATP–ADP *exchange reaction.* ADP labeled with either ^{14}C in the adenine ring or ^{32}P in the terminal phosphate group is rapidly incorporated into ATP in the presence of intact rat liver mitochondria (Wadkins, in press) or membrane fragments (Wadkins *et al.*, 1958: Bronk *et al.*, 1958), in the absence of net electron transport and in the absence of inorganic phosphate. Inorganic phosphate does not affect the rate of the exchange. This reaction is specific for the adenine nucleotides in the rat liver system.

In *intact* rat liver mitochondria this exchange reaction proceeds at a high rate and is stimulated by the presence of magnesium ions (Wadkins, in press). In the absence of added Mg^{2+} the ATP–ADP exchange reaction is inhibited over 90 per cent by 2×10^{-4} M DNP. On the other hand, the mitochondrial adenylate kinase, assayed by the incorporation of ^{14}C-labeled AMP into ADP in the presence of ATP, is completely unaffected by the presence of DNP. The inhibitory action of dinitrophenol on the ATP–ADP exchange is abolished by adding azide to the mitochondria. Azide itself does not inhibit the ATP–ADP exchange. The relationship of this exchange reaction to the coupling mechanism is also shown by the finding that the inhibitory action of dinitrophenol is highly dependent on pH and has two peaks of maximum inhibitory effectiveness at pH 6·5 and 7·5, in agreement with the interpretations of Myers & Slater (1957) on ATP-ase activity. The ATP–ADP exchange reaction in intact mitochondria is also inhibited by arsenate, an expected result in view of the arsenolysis of ATP described above. The ATP–ADP exchange reaction in intact mitochondria is inhibited up to 95 per cent by maintaining the carriers in the reduced state, again showing its relationship to the respiratory chain. Adenylate kinase is not inhibited by reducing the carriers.

In *digitonin fragments* the ATP–ADP exchange reaction shows similar properties (Wadkins *et al.*, 1958). It occurs in the absence of added phosphate, and it is not affected by additions of phosphate. The exchange reaction in freshly prepared fragments is sensitive to dinitrophenol, but it is significant that its DNP-sensitivity in digitonin fragments is generally speaking variable and usually less than in

intact mitochondria. In a number of representative preparations, the inhibition by 2×10^{-4} M DNP varied from 20 per cent up to 90 per cent, with the average approximately 65 per cent, whereas in intact mitochondria the inhibition is invariably above 90 per cent. Increasing the concentration of dinitrophenol does not increase the extent of inhibition. These findings suggested that some of the ATP–ADP exchange reaction in digitonin fragments is either extraneous to the coupling mechanism, or that it is "dislocated" from the coupling mechanism in such a way that it cannot interact with the earlier steps. The latter view is favored by the finding that on aging digitonin fragments the ATP–ADP exchange reaction does not suffer significant loss in activity, whereas the DNP-sensitive ATP–$^{32}P_i$ reaction is rather quickly inactivated over 24–48 hr, as is the capacity for oxidative phosphorylation. Over this time of aging, the DNP-sensitivity of the ATP–ADP is lost at the same rate as the loss of phosphorylation. DNP-sensitivity was also found to be abolished by the presence of either sodium azide or sucrose, in concentrations known to block respiratory energy coupling. These findings therefore indicate that the ATP–ADP exchange enzyme is not itself inhibited by dinitrophenol but that the exchange reaction stands in equilibrium with a preceding reaction of the coupling mechanism which contains the DNP-sensitive component. The connecting link between the two reactions is therefore inhibited by the presence of azide or sucrose, or is removed or inactivated by aging.

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It is quite significant that reduction of the respiratory carriers in digitonin preparations was found not to inhibit the ATP–ADP exchange reaction (Wadkins *et al.*, 1958) whereas when the same experiment is carried out with intact rat liver mitochondria the reaction is some 90 per cent inhibited (Wadkins, in press). This finding, together with the fact that DNP does not completely inhibit the ATP–ADP exchange reaction in digitonin fragments, suggests that in the latter, the ATP–ADP exchange enzyme is in part physically dislocated or detached from the normal reaction sequence. That is, the enzyme is present in the fragments and is quite active but not all of the enzyme molecules catalyzing this exchange reaction are so situated in the membrane that they can interact with preceding DNP-sensitive elements of the coupling mechanism.

The purified ATP–ADP *exchange enzyme.* Since the ATP–ADP exchange enzyme of rat liver mitochondria appears to be quite stable to aging and is readily dissociated from preceding steps of the coupling mechanism, either by means of inhibitors or by aging (Wadkins *et al.*, 1958), it seemed feasible to attempt the isolation of this enzyme in soluble form. This was accomplished by extracting acetone dried mitochondria or digitonin fragments with phosphate buffer, which extracted in soluble form nearly all the demonstrable exchange activity of the dried residue (Wadkins *et al.*, 1958). The soluble exchange activity in the extract was quite stable and survived extended dialysis. The dialyzed soluble enzyme has an absolute requirement for Mg^{2+} or Mn^{2+}, which are equally effective. The enzyme is also inhibited by –SH reagents: such inhibition is reversed by reduced glutathione or cysteine. In its soluble form the ATP–ADP exchange enzyme is not inhibited by DNP or other uncoupling agents. It is inhibited by very high

concentrations of thyroxine and other thyroactive compounds, but this effect appears not to be related to mitochondrial swelling induced by thyroxine.

Soluble extracts containing this enzyme were subjected to fractionation by means of isoelectric precipitation, ammonium sulfate fractionation and finally chromatography on DEAE-cellulose columns using as an assay system the rate of incorporation of labeled ADP into ATP. By these approaches purification up to 150-fold has been achieved by Wadkins.

Since the soluble enzyme, either in the crude or purified state, is insensitive to DNP, it no longer has distinctive properties relating it to the mechanism of oxidative phosphorylation. It is of course well known that a number of transphosphorylation enzymes are capable of catalyzing ATP–ADP exchange reactions. However, the known reactions of this kind could easily be excluded since the crude or purified enzyme did not require inorganic phosphate nor was it stimulated by it (distinguishing it from the enzyme of Chiga and Plant (1959)) nor could the reaction be stimulated by addition of other appropriate components, such as CO_2, succinate, CoA, acetate, creatine, AMP, and other relevant compounds (cf. Wadkins *et al.*, 1958). In addition the enzyme was not inhibited by incubation with avidin. The purified ATP–ADP exchange enzyme has no protein phosphokinase activity.

The ATP–ADP exchange enzyme has a rather low sedimentation constant and it appears probable that its mol. wt. is not much greater than 12,000. The presence of another protein component which has been extremely difficult to remove has delayed more exact measurements of mol. wt. The enzyme appears not to contain lipids, flavin, or cytochromes but does appear to contain a very small amount of bound nucleotide giving a small absorption shoulder at 600 mμ (Wadkins *et al.*, in press).

Reconstruction of DNP-sensitivity of ATP–ADP *exchange enzyme.* While every indication pointed to the probability that the soluble ATP–ADP exchange enzyme obtained from mitochondria or digitonin fragments was actually that moiety associated with the DNP-sensitive reaction, it was considered important to determine whether the soluble enzyme could be recombined again with digitonin fragments deficient in this enzyme in order to reconstitute a functional energy-coupling system.

The first criterion to be examined was that of DNP-sensitivity. If the soluble ATP–ADP exchange enzyme could be recombined with the membrane fragments in order to bring about a specific binding physically adjacent to and functional with preceding reactions of the coupling mechanism, then it could be expected that the specifically re-bound enzyme would again show DNP-sensitivity. This expectation was realized (Lehninger, 1960, in press; Wadkins *et al.*, in press). In Fig. 1 are shown typical data demonstrating recombination of a 30-fold purified preparation in such a manner that some 65 per cent of the added soluble ATP–ADP exchange activity was made DNP-sensitive, presumably by specific binding on the digitonin fragments. On the other hand, dinitrophenol sensitivity was not conferred on the soluble exchange enzyme when the digitonin particles were first aged to the point of complete inactivation of oxidative phosphorylation. Furthermore it was found that the recombination phenomenon did not occur in the presence of sodium azide; it

will be recalled that the reagent abolishes the DNP-sensitivity of the ATP–ADP exchange reaction in digitonin fragments or mitochondria.

When the soluble ATP–ADP exchange enzyme was incubated with digitonin fragments there was an increase in the amount of ATP–ADP exchange activity that

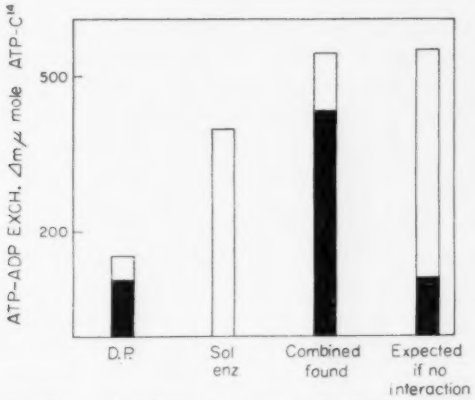

FIG. 1. Reconstruction of DNP-sensitivity of ATP–ADP exchange enzyme. Addition of soluble DNP-insensitive exchange enzyme to digitonin fragments (in which the ATP–ADP exchange is already partially sensitive to DNP) causes additivity of *total* exchange activity, but an increase of DNP-sensitivity far greater than expected from simple addition. The shaded portion is the DNP-sensitive fraction of the exchange activity.

could be sedimented with the digitonin particles. This observation thus provided direct evidence for physical binding of the soluble ATP–ADP exchange enzyme, presumably at unoccupied but still patent binding sites in the digitonin particles.

The ATP–ADP exchange enzyme is rather easily detached from digitonin fragments on incubation at pH 6·5 at 25°. High ionic strength, phosphate buffer, ATP and certain other additions were found to increase the dissociation of the enzyme in soluble form from the particles; at the end of such an incubation as much as 75 per cent of the enzyme could be detached under these rather mild conditions (Lehninger *et al.*, in preparation). Since the detached ATP–ADP enzyme is DNP-insensitive, these results thus explain the less than complete inhibition of the ATP–ADP exchange reaction by DNP in digitonin fragments, which was mentioned above.

An especially striking finding, which has led to a new direction of research, arose from a comparison of the rebinding of highly purified versus relatively crude ATP–ADP exchange enzyme preparations (Wadkins *et al.* (a), in press). It was found that enzyme preparations purified some 150-fold completely fail to recombine with digitonin fragments to restore DNP-sensitivity: on the other hand, the crude enzyme (20–30-fold purified) incubated with the same preparation restored dinitrophenol sensitivity. These findings suggested three alternative explanations, (a) the "wrong" ATP–ADP enzyme was purified by the procedure chosen, (b) the

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ATP–ADP exchange enzyme purified was the "right" enzyme, but suffered damage to a binding site necessary for attachment to the preceding enzyme, and (c) the ATP–ADP exchange enzyme is intact but a second factor necessary for its rebinding to the DNP-sensitive reaction was lost during purification. The last alternative seems to be most probable as indicated by the following experiments.

M-factor and the DNP-sensitivity of the ATP–ADP *exchange reaction.* During studies of the DNP-sensitivity of the ATP–ADP exchange reaction in digitonin fragments, it was found that certain soluble protein fractions obtained from acetone-dried mitochondria increased the DNP-sensitivity of the ATP–ADP exchange reaction of fresh digitonin fragments. This effect was made the basis of a quantitative assay procedure and the protein factor involved was purified by salt fractionation. This factor, called M-factor (Wadkins *et al.* (b), in press), has little or no ATP–ADP exchange activity itself but increases the dinitrophenol sensitivity of the ATP–ADP exchange in digitonin fragments, as shown in Fig. 2. M-factor

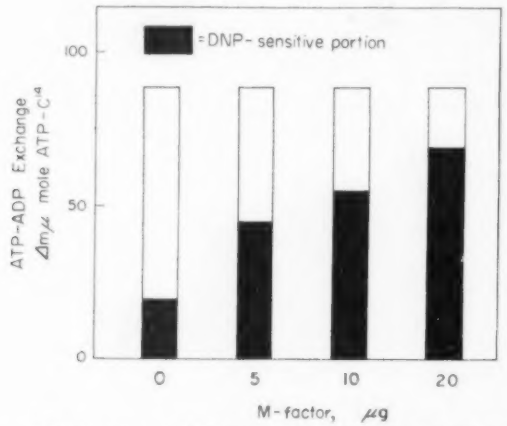

FIG. 2. Action of M-factor. Addition of M-factor to digitonin fragments does not affect total ATP–ADP exchange activity, but increases the fraction sensitive to 2×10^{-4} M DNP (shaded portions).

activity is heat-labile and is not given by other proteins such as serum albumin, or by other mitochondrial fractions, including those containing adenylate kinase. M-factor has been purified approximately 60-fold and at this stage contains no ATP-ase or DNP-stimulated ATP-ase activity, no adenylate kinase, little or no ATP–ADP exchange activity nor is it capable of catalyzing the ATP–$^{32}P_i$ exchange reaction.

Other activities of M-factor. Recent experiments by Wadkins and Gregg in this laboratory show that M-factor preparations of 60-fold purification are capable of stimulating the ATP–$^{32}P_i$ exchange reaction in digitonin fragments as much as 10-fold. Furthermore ATP-ase activity of digitonin particles is also greatly stimulated by M-factor; this effect requires the presence of Mg^{2+} and DNP.

These are the properties expected of an intermediate enzyme of oxidative phosphorylation capable of serving as the connecting link between the ATP–ADP exchange reaction and the earlier DNP-sensitive reaction component.

For these reasons it is tentatively suggested that M-factor may be an intermediate enzyme of the coupling mechanism as indicated in the following reaction sequence, in which M replaces the X of the formulation of reactions (1)–(5), and E is the ATP–ADP exchange enzyme:

$$(6)\ \text{Carrier}_{ox} \sim M + P_i \rightleftharpoons P \sim M + \text{Carrier}_{ox}$$

$$(7)\ P \sim M + E \rightleftharpoons E \sim P + M$$

$$(8)\ E \sim P + ADP \rightleftharpoons ATP + E$$

Alternatively it is possible that M-factor represents a specific "cementing" substance, without enzymatic activity, necessary for holding together the molecular entities involved in the DNP-sensitive reaction and the ATP–ADP exchange reaction (cf. Lehninger, 1960, in press; Wadkins (b), in press).

Tests of protein components by restoration of oxidative phosphorylation. The ultimate criterion for the relevance of any given reaction component or enzyme to the energy-coupling mechanism must be its ability to increase the P : O ratio in a mitochondrial or submitochondrial preparation in which the given component has been removed. When membrane fragments are prepared with an excess of 2% digitonin, the P : O ratios (β-hydroxybutyrate as substrate) are substantially decreased, indicating partial loss (or inactivation) of intermediate catalytic components (Lehninger, 1960, in press). When preparations containing M-factor are added to such fragments, a significant increase in P : O ratio occurs. Similarly, addition of purified ATP–ADP exchange enzyme to such systems causes a significant increase in P : O (Δ 0·4–0·6). These experiments are very encouraging for the view that these enzymes are components of the energy-coupling mechanism. However, the test system used in these studies is unsatisfactory, since the preparations have some phosphorylating activity at all three sites, but to different degrees. It would be most desirable to use a test system in which a single phosphorylation site could be separated for reconstitution of net phosphorylation.

Relationship of M and E to heart ATP-*ase.* Recently Pullman and Racker and their colleagues have described a soluble DNP-stimulated ATP-ase from heart mitochondria which can restore the P : O ratio of mechanically treated heart mitochondria, from about 0·0 to as high as 0·8 (Pullman *et al.*, 1960; Penefsky *et al.*, 1960). The enzymatic relationship of M-factor and the ATP–ADP exchange to this ATP-ase is a question of some interest. They have found that the heart ATP-ase does not show either ATP–ADP exchange activity or ATP–$^{32}P_i$ exchange activity. Furthermore it does not catalyze the ATP–ADP–$^{32}P_i$ exchange and thus is not identical with the enzyme of Chiga & Plaut (1959). They have also made the finding that neither the ATP–ADP exchange enzyme (Wadkins *et al.*, 1955) nor the ATP–ADP–$^{32}P_i$ exchange enzyme of rat liver (Chiga *et al.*, 1959) can replace the heart ATP-ase in restoring the P : O ratio in heart mitochondria.

While these findings suggest superficially that there are no common denominators shared by the heart ATP-ase and the liver enzymes (M-factor and ATP–ADP enzymes), this conclusion may be premature. The species and organ differences between the test systems cannot be disregarded, and it is also to be noted that the phosphorylation sites in the respiratory chain which are partially or completely restored in the heart and liver systems respectively are not known with certainty and may not be identical in mechanism or in the molecular and ultrastructural organization of the lipoprotein membranes in which the energy-coupling reactions occur. Perhaps the most significant point is the failure of Pullman and Racker to find the ATP–ADP exchange reaction in the heart ATP-ase, in view of our recent finding (Lehninger, unpublished) that rat heart mitochondria contain a DNP-sensitive ATP–ADP exchange reaction in addition to DNP-insensitive adenylate kinase activity. There are significant differences in the ATP-ase activity in the two organs, and in their sensitivity to inactivation, which suggest that the postulated "water sites" of the ATP-ase activity formed in the inactivation of the coupling mechanism may differ in affinity in the two systems. Finally, occurrence of the ATP–ADP exchange is a reflection of the relative rates and affinities of ADP with the enzyme, in relation to affinities to other components, whether H_2O, P_i, ATP, or some other molecule is the coupling sequence. The widely variable exchange ability of labeled nicotinamide into DPN catalyzed by DPN-ases from different species and organs (Zatman *et al.*, 1954) is possibly an analogous case.

Relationship between mitochondrial swelling and contraction and oxidative phosphorylation. In recent reviews (Lehninger, 1960, Lehninger, a, b, c in press) experimental evidence has been summarized relating the mechanism of mitochondrial swelling and contraction with the mechanism of oxidative phosphorylation. Respiration is required in order for mitochondria to be susceptible to swelling agents such as phosphate, thyroxine, Ca^{2+}, and U-factor. Mitochondrial contraction, on the other hand, requires the institution of phosphorylating respiration but can also be induced by the presence of ATP alone, in the absence of respiration. Contraction induced by ATP is not inhibited by dinitrophenol, as is mitochondrial swelling, but is on the other hand blocked by the uncouplers azide and sucrose. These findings thus show that swelling and contraction of mitochondria are brought about by action of intermediate enzymes of energy coupling in the respiratory chain. Accordingly we have postulated that the intermediate enzymes of respiratory energy coupling are "mechanoenzymes" (Lehninger, 1959). Recently we have found that swelling of mitochondria induced by GSH is accompanied by leakage of a soluble protein into the medium. This protein is necessary for contraction of mitochondria by ATP, in such a fashion that the concentration of this protein (C-factor) determined not the *rate*, but rather the *extent* of contraction.

C-factor has recently been purified by Neubert, to a level of about 100-fold. While it is not yet a homogeneous protein, it has been found that such preparations of C-factor contain considerable M-factor activity. Thus it appears possible that the C-factor protein, active in the mechanochemical contraction of mitochondria, may be identical with an intermediate enzyme of the energy-coupling mechanism

of the respiratory chain. Fig. 3 shows a schematic suggestion for the function of intermediate enzymes of the energy-coupling mechanism as "mechanoproteins" in bringing about ATP-induced contraction of the mitochondrial membranes.

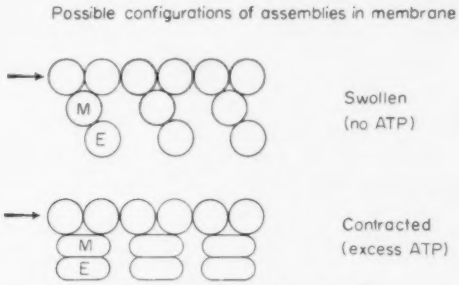

FIG. 3. Schematic representation of postulated action of energy-coupling enzymes as "mechanoenzymes" in controlling state of swelling or contraction of mitochondrial membrane in which the respiratory assembly is embedded.

Last, it is necessary to point out the striking similarities between the enzymatic and mechanochemical properties of actomyosin and the energy-coupling mechanisms in the mitochondrial membranes. These similarities, which include multiple pH optima, rather similar to effects of heavy metals and azide, stimulation of ATP-ase by DNP, ^{18}O-exchange between P_i and H_2O, involvement of divalent cations, and characteristic ionic strength effects, among others, suggest that these two contractile systems derived from a common precursor catalyst in the course of molecular evolution and adaptation.

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SOME OBSERVATIONS ON THE BASIC PRINCIPLES OF DESIGN IN PROTEIN MOLECULES

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The last stage in the biosynthesis of an enzyme involves the intramolecular coiling and folding of the extended polypeptide chain into a configuration that embodies a unique active center. The arrangement of amino acids along the chain must be such that the final tertiary structure is assumed with a high degree of reproducibility and must also contain information governing the correct pairing of half-cystine residues and the formation of other stabilizing covalent cross-linkages. Experiments on ribonuclease are discussed which suggest that all the information for the production of the fully cross-linked and folded molecule is contained in the amino acid sequence alone.

In these experiments, the four SS bonds of RNase were reduced to yield a derivative containing eight SH groups. The oxidation of this reduced derivative to yield native enzyme is quantitative under proper conditions of protein concentration and pH. Various substances and environmental conditions were tested for their ability to interfere with normal pairing of half-cystine residues, thus furnishing information on the nature of the interactions that govern this process.

It is suggested that the process of mutation and natural selection has lead to the "design" of amino acid sequences so uniquely constructed that the formation of active centers and three-dimensional conformations follow automatically and reproducibly without additional genetic information.

To SPEAK of "the design of protein molecules" is to imply some purposeful choice of alternatives in the course of evolution. This vitalistic view is certainly incorrect —evolution is the result of a completely random set of mutational events, the results of which may or may not be compatible with environmental conditions. The use here of the word "design" is intended only to imply the empirical correlation of *observed* structure with *observed* properties and is not meant to suggest that Nature can make rational choices. The understanding of at least a few proteins in terms of organic chemistry has now progressed to a stage that permits some preliminary discussion of the intramolecular interactions governing secondary and tertiary conformation in these molecules.

The biosynthesis of a protein molecule must conform to an extremely complex and interlocking set of requirements. The molecule must, first of all, be so constructed that it can carry out its biological function in smooth co-operation with other proteins characteristic of the particular species and cell type. As has become abundantly evident in recent years, enzymatic activity is dependent on unique three-dimensional configurations which may involve amino acid residues widely separated along the polypeptide chains. The biosynthetic process must, therefore,

follow a set of specific directions for the linear alignment of amino acid residues which are also compatible with the ultimate spatial relationships assumed by the individual side-chain functional groups in the folded protein molecule. (One might describe this, anthropomorphically, as "looking ahead" on the part of the biosynthetic machinery. Actually, what we observe here is the result of continuous natural selection for optimal function of the entire organism.)

A second requirement of the genetically determined sequence must be the presence of correctly distributed chemical "information" along the chain to determine the pairing of half-cystine residues in disulfide bonds. To anticipate later parts of this discussion, our own experiments on ribonuclease, which I shall discuss in more detail below, indicate that such pairing is determined by a wide variety of intramolecular interactions and that the whole process of the formation of covalent cross-links proceeds with a very high degree of reproductibility. Furthermore, our experience with this protein suggests that its entire secondary and tertiary structure can form spontaneously, under quite simple environmental conditions, without the necessity for additional genetically transmitted information. It is tempting to postulate that these results will be of general applicability to the biosynthesis of other proteins, particularly since some suggestive results along these lines have already been obtained on egg-white lysozyme and, quite recently, on insulin. The preceding discussion assumes that polypeptide chain synthesis utilizes *cysteine* rather than *cystine*. The efficiency of oxidation of the reduced chain of RNase gives some indirect support to this assumption.

THE MOLECULAR BASIS OF FUNCTION IN PROTEINS

It is now clear that the complete structures of many proteins are not essential for their biological activities. A number of enzymes, hormones and immunochemically active protein molecules have been shown to retain normal, or nearly normal, function after considerable degradation by proteolytic or chemical agents. Ribonuclease, whose covalent structure (Hirs *et al.*, 1960; Spackman *et al.*, 1960; Redfield & Anfinsen, 1956) is shown in Fig. 1, is an example of such a protein. It contains a number of residues which can be modified or removed without inactivation. Conversely, other residues appear to be essential. The case of ribonuclease is a particularly difficult one since residues that are widely separated in the sense of a linear sequence appear to be involved, co-operatively, in catalysis (Richards & Vithayathil, 1959; Vithayathil & Richards, 1960; Anfinsen, 1956; Gundlach *et al.*, 1959; Stark *et al.*, 1961; Barnard & Stein, 1959; Stein & Barnard, 1959; Klee & Richards, 1957; Taborsky, 1959; Hirs *et al.*, 1960). Furthermore, the detailed nature of the interactions contributed by those side-chain functional groups that *can* be modified without inactivation is unknown. I shall mention only a few instances here since the subject is also considered by other contributors to this symposium. In studies similar to those described earlier by Becker & Stahmann (1953), Stracher & Becker (1959), Fraenkel-Conrat (1953) and others, we have recently been able to mask nearly all of the ten epsilon amino groups of ribonuclease with chains of polyalanine containing as much as five or six alanine residues

(Haber *et al.*, 1961). The alaninated molecule is still fully active in spite of the fact that approximately one-third of the entire ribonuclease derivative is made up of alanine. One can only guess that the exposed amino groups on the native molecule

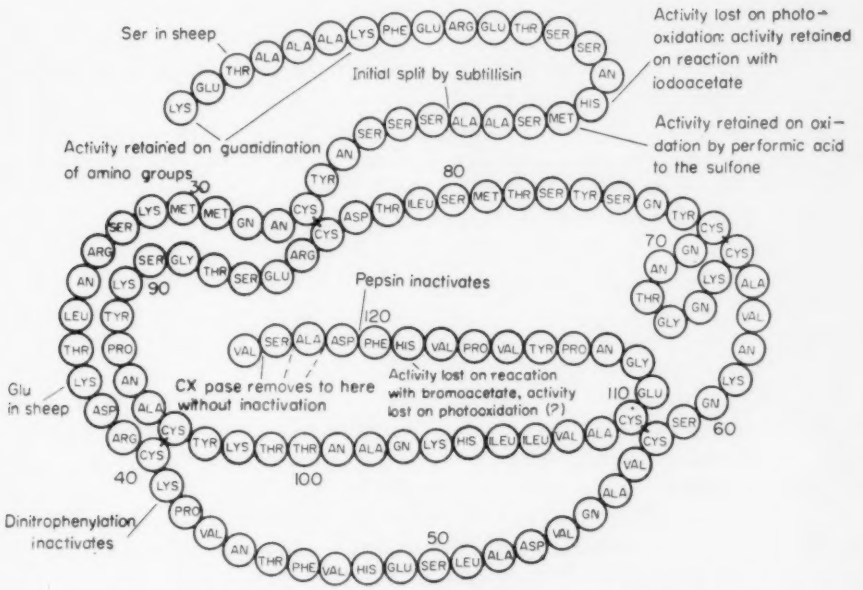

FIG. 1. The covalent structure of bovine pancreatic ribonuclease.

exert their effect on three-dimensional structure, and on activity, not because of rigid spatial orientations, but through their contribution to the net charge of the protein. Along similar lines, Hirs and his colleagues (1960) have recently shown that the charge on a single epsilon amino group (residue no. 41) may be masked by dinitrophenylation with complete inactivation.

One sort of interaction appears to be invariably associated with an active structure in this enzyme, namely that involving three of the six tyrosine residues in the enzyme. The characteristic wavelength of maximum light absorption at 277·5 mμ for native RNase, and for enzymatically active derivatives of the protein, is shifted to 276 mμ following any treatment that results in inactivation (Sela & Anfinsen, 1957; Sela *et al.*, 1957). It seems very likely, therefore, that the active configuration of the protein requires orientation of these tyrosine residues in such a way that resonance in the benzene rings can be modified by unique interactions of the phenolic hydroxyl groups, possibly with certain electro-negative groups elsewhere in the molecule. The shift in the absorption maximum caused by strong urea solutions is, incidentally, reversed by the addition of very small amounts of a number of polyvalent anions to such solutions, suggesting that the critical tyrosine configurations are in some way favored or determined by stabilizing electrostatic

forces (Sela & Anfinsen, 1957; Sela *et al.*, 1957). The importance of such electrostatic effects has also been suggested by various studies on the influence of orthophosphate and cytidylic acid on the specific reactivity of a single histidine residue in RNase to iodoacetic acid (Stark *et al.*, 1961; Barnard & Stein, 1959; Stein & Barnard, 1959).

Certain other biologically active proteins have proved to be much more easily degraded than RNase because they exist in the form of polypeptide chains without covalent cross linkages. A dramatic example is papain, whose chain of 180 residues was degraded, without inactivation, to approximately 40 per cent of this length by treatment with leucine aminopeptidase (Hill & Smith, 1960). In a similar way, ATCH was shown by Bell and his colleagues (1956) to retain activity after removal of more than a third of the total amino acid sequence from the carboxyl-terminal end of its chain of 39 residues. The recent organic synthesis of an active molecule identical with the first 24 residues of the structure, by Klaus Hofmann and his collaborators, confirms the results obtained by degradation. These studies also furnish an important and reassuring test of the adequacy and dependability of the methods that have been employed for the determination of polypeptide structure.

In studies of the effects of degradation on enzymes, the tests for function have involved *in vitro* assays and one cannot, therefore, state with certainty that these same derivatives would have acted normally *within cells*. However, on the basis of studies of species variation in the structures of biologically active proteins, considerable changes in covalent structure appear to be permissible without significant alteration in efficiency or mode of action. The proteins of any particular species are characteristic of that species and their structures reflect a line of genetic information that has branched off from the main stream, becoming insulated from the hereditary characteristics of other independent lines. The "active center" of homologous proteins might remain relatively unchanged from species to species in accordance with the structural requisites of the function in question, but the species specific part of the structure not directly associated with function might be varied enormously as the result of mutation and selection within any isolated gene pool.

Figure 1 shows one example of species variation, the differences between the ribonucleases isolated from the pancreatic tissues of cows and sheep (Åqvist & Anfinsen, 1959; Anfinsen *et al.*, 1959). Residue no. 37, which is lysine in the bovine molecule and glutamic acid in ovine ribonuclease, would appear to be an unlikely candidate for a critical role in catalysis because of the marked change in charge introduced at this locus of the protein by the replacement. A similar example is found in studies on the various insulins, where the structures of this protein from a wide variety of species have been examined (Harris *et al.*, 1956; Ishihara *et al.*, 1958). All of the observed variations have been shown to occur within one restricted portion of the B chain, a part of the molecule that one might therefore conclude is not uniquely "designed" on functional grounds, but rather is related to the species—specific characteristics of the insulin molecule. Another well-known example of the species variation involves the cytochrome *c* molecule.

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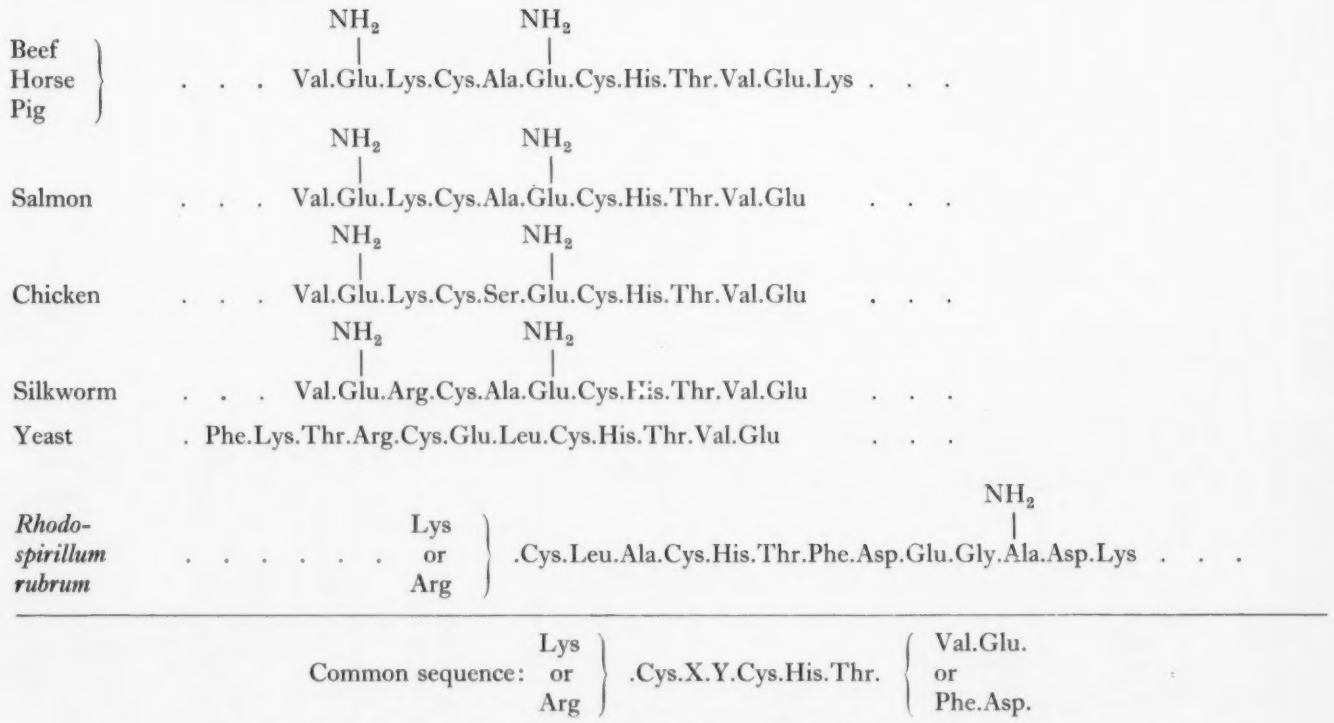

FIG. 2. Variations in the sequence of the polypeptide chain of cytochrome *c* from species to species. From H. Tuppy, *Symposium on Protein Structure* (A. Neuberger, editor), John Wiley, 1958.

In these comparative structural studies Tuppy (1958) has emphasized the relative *absence* of change in a part of the sequence that is very closely associated with the heme prosthetic group. Whereas the rest of the molecule might undergo considerable variation (this has not yet been sufficiently documented to permit useful discussion), an unchanged sequence in the vicinity of the "active center" has been selected for quite stubbornly, except for certain functionally homologous replacements such as arginine for lysine or phenylalanine for valine.

Perhaps the most convincing example of the repeated use by Nature of a successful bit of design in protein structure comes from numerous studies on the amino acid sequences in the vicinity of the active centers of various hydrolytic enzymes. A number of such enzymes, catalyzing quite different reactions, have been shown to contain the same, or nearly the same, sequence around a critical di-isopropylfluorophosphate-sensitive serine residue (summarized in Boyer, 1960). The serine residue in this sequence, which has the general form $\left(\begin{matrix} \text{Glu.} & \text{Gly.} \\ \text{Gly. or .Ser. or} \\ \text{Asp.} & \text{Ala.} \end{matrix} \right)$, is almost certainly involved in the covalent changes that take place during the catalytic process since its hydroxyl group must be free to permit enzyme action to proceed. Interesting studies on model compounds (Bernhard, 1959) based on the sequence Asp. Ser. have demonstrated that this combination of amino acids has very unusual properties in being able to form intramolecular configurations of high intrinsic instability which may be related to the intermediates formed in hydrolytic enzymes during the catalytic process.

DETERMINATION OF SECONDARY AND TERTIARY STRUCTURE IN PROTEINS

The discussion to this point has been directed mainly at lending support to the general thesis that the structure of any enzyme may be divided into two parts, one part essential for the actual performance of a catalytic act involving covalent changes and the other for endowing the molecule with its specific characteristics. This hypothesis includes the supposition that the former category is less subject to change during evolution than the latter, because of the more exacting specifications of substrate-fit required for uniform function in all organisms (Anfinsen, 1959). The active center of an enzyme must be stabilized by the complex set of interactions which we term secondary and tertiary structure, although it is clear that this spatial organization may be achieved in a variety of ways. For example, the lysozymes of bird egg-white and of bacteriophage T4 are both capable of hydrolyzing similar bonds in certain polysaccharides in spite of the fact that the bird protein is cross-linked through numerous disulfide bridges, while that from phage, which appears to contain no such bonds, must depend upon noncovalent bonds for the maintenance of its three-dimensional integrity (Dreyer & Crumpton, unpublished results). I should like now to consider an experimental

approach which we feel can yield direct evidence on the nature of the interactions that determine catalytically active configurations.

The four disulfide bonds of bovine pancreatic ribonuclease can be reduced with mild reducing agents to yield a product which contains eight sulfhydryl groups and appears to be free of other covalent change (Sela *et al.*, 1957; White 1960, in press; Anfinsen & Haber, in press). By exposing a dilute solution of the reduced polypeptide chain to molecular oxygen at approximately neutral pH, the reduced derivative is converted to the original native form in essentially quantitative yields (White, in press; Anfinsen & Haber, in press).

TABLE 1—OXIDATION OF REDUCED RNase AT VARIOUS PROTEIN CONCENTRATIONS

Concentration of reduced protein mg/ml	% Yield of soluble protein	Activity of soluble fract. % of equivalent conc. of native RNase	% Regeneration of activity
7·0	27	31	8
4·8	42	70	29
2·3	87	75	65
0·9	100	77	77
0·35	100	94	64

The reduced protein was freed of reagents on Sephadex G-25 (Anfinsen & Haber, in press). The protein in the column effluent, after appropriate dilution, was adjusted to pH 8·5 and allowed to stand without stirring at room temperature for 20 hr at which time the proportion of soluble protein and the enzyme activity of this fraction were estimated.

A variety of physical and immunochemical procedures have been employed to test the nativeness of the regenerated material and have thus far shown no detectable differences. A kinetic study of the oxidation process (unpublished data) has shown that both the disappearance of SH groups and the increase in specific optical rotation of the material begin at once and follow the same smooth exponential curve. On the other hand, the appearance of enzyme activity against RNA or cyclic cytidylic phosphate substrate and the re-establishment of the characteristic spectral shift of the native enzyme do not occur until a considerable lag period (as much as several hours) has elapsed. The lag is unexplainable at the present time, but might involve either a uniform population of molecules undergoing stepwise disulfide bridge formation or, alternatively, the formation of "incorrectly" cross-linked molecules in the early stages which then undergo subsequent internal rearrangement to yield the native form.

These experiments can only indicate that the complete "information" for the formation of native secondary and tertiary structure in ribonuclease is contained in the extended amino acid sequence and introduce a considerable simplification into our thinking about the latter stages of the biosynthesis of this protein molecule.

Admittedly, the process as it occurs within the cell might be under additional regulation to control its efficiency and metabolic integration. The formation of three-dimensional shape is, at any rate, a thermodynamically spontaneous process and, from the yields observed in the *in vitro* system, would appear to function with little, if any, error.

It is unfortunate that we cannot as yet discuss the possible generality of this process for other proteins. Preliminary experiments on fully reduced egg-white lysozyme (F. H. White, unpublished data) have yielded enzymatically active material, but the chemical nature of the re-formed material has not been studied in any detail. Attempts to regenerate native insulin by recombination of the two separated, reduced chains has not met with success, although Dixon & Wardlaw (1960) have recently reported that approximately 2 per cent of native hormonal activity may be re-formed. This figure, however, is approximately that (6·7 per cent) to be expected for random recombination of the six SH groups involved (Sela & Lifson, 1959; Kauzmann, 1959). It seems possible that only those proteins that consist of a single chain may be subject to the sort of mechanism that appears to operate for ribonuclease. The interesting suggestion has been made that insulin is actually synthesized in a cross-linked precursor form, with a single longer chain which is cleaved specifically during an activation step (W. Dreyer, personal communication).

The 124 amino acid residues which form the RNase chain are not all essential for the refolding process. We have recently shown that the RNase derivative, RNase-S-protein (Richards & Vithayathil, 1959; Vithayathil & Richards, 1960), prepared from RNase by limited digestion with subtilisin, can also undergo reversible reduction-reoxidation of its four SS bonds (Haber & Anfinsen, in press), This derivative contains only 104 residues, in which must reside the required information for directing the process. The oxidation is somewhat less efficient with this material and considerable increase in yield occurs when the oxidation is carried out in the presence of the other fragment of RNase produced by the subtilisin digestion, the 20 amino acid residue, RNase-S-peptide. However, the major effect of this addition appears to be the prevention of the rather considerable aggregation that occurs with reduced RNase-S-protein in neutral or slightly alkaline solutions.

SPECIFIC INTERACTIONS GOVERNING REFOLDING

The various side-chain interactions that contribute to the control of SH-pairing during oxidation may be examined by studying the inhibitory effect of physical and chemical agents on the process. Such studies have shown that proper control depends upon the combined action of electrostatic and hydrogen bonds. Direct evidence for involvement of hydrophobic and Van der Waals interactions is not yet available. The importance of electrostatic charge distribution is indicated by the fact that the fully reduced RNase chain is relatively stable at pH 3·5 and that, when very long time periods are allowed for complete oxidation of SH groups to occur at this pH, an inactive product is obtained (White, 1960; Haber & Anfinsen, in press).

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Evidence on the role of exact spatial positioning of charged groups in refolding comes from the experiments, mentioned in the introduction, where epsilon amino groups of lysine were covered with polyalanine chains. By treatment of native RNase with D,L-N-carboxyalanine anhydride various derivatives were obtained which could be chromatographically separated both from native RNase and D,L-polyalanine (Haber *et al.*, 1961). These were essentially fully active on a molar basis. The most heavily alaninated derivative, containing only 2–3 of the original ten epsilon amino groups and consisting of alanine to the extent of more than 40 per cent, yielded 100 per cent regeneration of enzyme activity when dilute solutions of the inactive, reduced form were exposed to oxygen at pH 8. Since the positive charges on the terminal alanine moieties are, on the average, removed from their original location on the epsilon amino group by some 15–20 Å, their exact spatial orientation with respect to the rest of the molecule does not seem to be critical.

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The native structure of RNase is strongly dependent on an intact system of hydrogen bonds as shown by the marked alterations in its optical rotatory, spectral and hydrodynamic properties by such agents as urea or guanidine HCl. Reagents that cause the rupture of hydrogen bonds also prevent the correct pairing of half-cystine residues during oxidation of the reduced chain (Haber & Anfinsen, in press). The product formed is a mixture of randomly cross-linked molecules with a specific enzyme activity corresponding to the presence of about 1 per cent native enzyme (the amount predicted on a purely statistical basis, assuming that all four disulfide bonds are essential for activity, would be 0·95 per cent). The optical rotatory properties and intrinsic viscosity of the material formed in the presence of urea are very similar to those of the native enzyme. However, its spectrum does not show the shift towards higher wavelengths that has uniformly been observed for derivatives of the enzyme that are active.

It is of special interest that the internal coiling as indicated by optical rotatory measurements is not markedly different in amount from that present in the native protein. This finding supports the hypothesis that the formation of helical coiling is spontaneous when this is sterically possible. Furthermore, the process would not appear to be dependent upon the presence of *particular* stabilizing cross-linkages, but only require that the random chain be capable of forming arbitrary centers of rigidity, thus avoiding the "unravelling" effect postulated by Schellman & Schellman (1958) at free chain termini.

The absence of significant covalent change other than incorrect SH pairing is demonstrated by the fact that the product formed in urea may be reduced a second time and then made to refold with good regeneration of activity by oxidation under standard optimal conditions (Haber & Anfinsen, in press).

The interactions of phenolic hydroxyl groups that appear to characterize the active configuration of RNase also play a marked role in the correct conversion of the reduced, extended chain to the native cross-linked form. A variety of analogs of tyrosine have shown a marked capacity to interfere with normal pairing of half-cystine residues. Of those listed in Table 2, only the ones bearing a phenolic hydroxyl group and lacking a charged group on the benzene ring were effective.

Amino acid copolymers have also been tested for their ability to alter the normal pattern of oxidation. A copolymer (degree of polymerization *ca.* 100) containing equivalent amounts of the two amino acids, glutamic acid and tyrosine,

TABLE 2—THE EFFECT OF VARIOUS COMPOUNDS ON REGENERATION OF ACTIVITY OF REDUCED RNase*

Compound	Concentration† (molar)
Effective	
Phenol	0·1
α-Napthol	0·05
p-Chlorophenol	0·05
p-Cresol	0·1
p-OH phenyl acetic acid	0·1
3-OH pyridine	0·1
Ineffective	
Dioxane	1·0
Methanol	1·0
p-OH Benzoic acid	1·0
p-Nitrophenol	(Saturated 0·15)
Benzyl alcohol	(Saturated 0·2)
Cyclohexanol	(Saturated 0·2)
Phenylacetic acid	0·3

* Oxidations were carried out with 0·01% reduced RNase at pH 8·0 in tris-acetate buffer. Control oxidations yielded 90–100 per cent regain of activity.

† Concentration of effective inhibitors is that at which less than 1 per cent of enzyme activity is regained; of ineffective inhibitors, that at which 50 per cent or more enzyme activity is regained.

and consequently rich in the sequences Tyr. Glu. and Glu. Tyr., completely inhibited correct oxidation at levels of 0·4 mg/mg reduced RNase (Haber *et al.*, 1961). Polyaspartic acid was ineffective. The striking potency of the phenol moiety when immobilized in polymer form suggests that such a macromolecule may undergo multipoint attachment to the RNase derivative and thus yield high local concentrations of competitor not attainable by the smaller compounds listed in Table 2. The 1 : 1 copolymer is, incidentally, an extremely potent inhibitor of RNase activity (M. Sela, unpublished). However, polymers containing one tyrosine residue per four and per nine glutamic acid residues were not active at these concentrations.

CONCLUSIONS

Most biologists accept, as a truism, the statement that living organisms are constructed from chemical components whose structures and properties are so ingeniously "designed" that significant deviations from normal, smooth operation

are extremely rare. It is only in the past few years, however, that we have been able to appreciate this statement when applied to macromolecules. The studies showing that such complex molecules as tobacco mosaic virus (Fraenkel-Conrat & Singer, 1959), DNA (Marmur & Lane, 1960; Doty, 1960) and hemoglobin (Itano & Robinson, 1959; Jones *et al.*, 1959) may be re-formed after disruption of their native conformations, begin to suggest that, even with these large molecules, "design" is such as to leave very little to chance. Indeed, it becomes tempting to believe that the phenomenon known vaguely as "denaturation" is only an apparent one in the case of substances that have not suffered *covalent* modification. The inability to regenerate biological activity in proteins that have been denatured by agents that have not caused covalent change may simply reflect our ignorance of the proper laboratory tricks.

The constant theme throughout all of our considerations of protein structure and function must be the basic evolutionary process of random mutation and natural selection. To satisfy those who are evolutionary purists, it might be added that such selection is, of course, at the organism level and not at the level of molecules, although the act of mutation undoubtedly exerts its effect on the phenotype through modification of the structure of an individual protein molecule at the command of an altered gene. The studies on ribonuclease summarized here will, hopefully, be a prototype of similar studies on other proteins that might enable us to gain some understanding of the process by which the information coded into the genetic material of cells is translated into the unique structure and function that characterizes protein molecules.

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BIOCHEMICAL GENETICS AND EVOLUTION

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IT SEEMS most appropriate to introduce this session on the genetic basis of evolution first by summarizing the broad aspects of evolutionary theory from the genetic viewpoint, as involving gene mutation, recombination, and selection; second, by outlining present concepts of the nature of the gene in terms of molecular genetics; and then by looking at the problem of gene action and regulation at the molecular level. Finally, we will examine the role of selective pressures, operating on genetic material at the molecular level, and consider briefly the origin of new genetic material and gene functions.

No one has yet definitely observed evolution in progress in the laboratory, and the following broad statement may be considered too glib and sweeping in light of the views of students of evolution such as Goldschmidt. Nevertheless, a concept of the mechanism of evolution as involving primarily mutation and selection, essentially as first outlined by Darwin, seems in best accord with the evidence available. According to this hypothesis the genetic material in any organism defines its range of potentialities. Random changes or mutations in this genetic material occurring independently of environmental factors result in corresponding changes in the potentialities of the organism. The mutant genetic material may either be eliminated or maintained, depending on the survival value of the new phenotype for the organism in a particular environment. The flexibility of a species in surviving in, and evolving to meet, a changed environment depends on the frequency of mutation, and on the available reservoir or "gene pool" of varied genetic material. The latter is increased by biological developments such as the existence of multinucleate haploid forms in bacteria and fungi, and diploidy in higher plants and animals, both of which permit the survival of mutant genes. Evolutionary flexibility is also increased by biological mechanisms of gene recombination such as transformation or transduction in bacteria, or as sexual recombination either in haploid bacteria or in diploid higher forms.

Another important factor in evolution is that of isolating mechanisms, either reproductive, geographical, or related to population size, in which the gene pool available for recombination and selection may be limited by chance rather than by selective survival value.

As a consequence of biological and chemical advances over the past decade or so, it is now widely accepted that the basic genetic material in all forms of life is nucleic acid—generally DNA. In certain viruses, however, plant, animal and, most recently discovered, bacterial, the nucleic acid is of the RNA type. It appears that all genetic information is expressed directly via protein amino acid sequences and

is coded into the base pair sequence of specific double stranded helical molecules of DNA, or of single stranded molecules of DNA, or RNA in some instances. Mutation is interpreted as involving alterations in specific base pairs in the sequence, and gene replication as a molecular mechanism based on the Watson–Crick DNA model. We now know a great deal about the enzymatic biosynthesis of DNA (Kornberg, 1960) and of RNA (Ochoa, 1960), and realize that DNA serves as a specific in the synthesis of new DNA molecules with base sequences specifically determined by the sequence in the primer DNA. We know from studies on labelled DNA and chromosomes that a semi-conservative mechanism of DNA replication, consistent with the predictions from the Watson–Crick model, is involved (Taylor, 1960; Meselson & Stahl, 1958).

We have a much clearer concept of the nature of the mutation process, to a considerable extent as the result of experiments on mutation induced as a consequence of the introduction of base analogues into replicating DNA (Freese, 1959; Zamenhof & Gribhoff, 1954). Freese, for example, has suggested on the basis of differences in revertibility that mutations may result from the replacement of a purine by a pyrimidine, or vice versa, or from the replacement of a base, either purine or pyrimidine, by another of the same type. Changes in base pair sequence may result from incorporation of a different base during the copying process, as a consequence of introduction of an analogue such as Br-deoxyuridine or of a substitution of a purine for a pyrimidine with the replacements involving alterations in H-bond configuration. Physical treatments which change the tautomeric base and hence the H-bonding forces or configurations presumably also are mutagenic.

In the case of tobacco mosaic virus (TMV), the work of Schuster & Schramm (1958) and Tsugita & Fraenkel-Conrat (1960) has provided evidence that alterations of bases in the genetic RNA, brought about by nitrous acid, result in detectable mutation of the virus. Since, under the conditions employed, the effect of nitrous acid is apparently limited to the conversion of an amino group on a base to a keto group, the resulting mutation can be attributed to the consequent change in base sequence in the RNA molecule.

It seems probable that the detailed correlation of nucleic acid base sequence with protein amino acid sequence, needed for solution of the code which related nucleic acid and protein structures, will be reached sooner with viruses than with more complex systems. This is especially true of RNA viruses, notably TMV, in which the 158 amino acid sequence has been completely established (Anderer *et al.*, 1960; Tsugita *et al.*, 1960), and in which the 6400 bases in the RNA are more readily accessible to sequence analysis than in DNA. Even here only a fraction of the RNA seems to be related to the protein amino acid sequence.

In DNA controlled systems, the desired correlation can as yet be carried only as far as to establish that the effect of gene mutation in some instances is to cause the replacement of one amino acid in the β-chain of hemoglobin by another amino acid (Hunt & Ingraham, 1959; Hill *et al.*, 1960). In other systems in which more sophisticated genetic analysis and experimentation are possible, protein

changes can be correlated with changes in the gene as defined in terms of units of mutation, of recombination, or of function (Benzer, 1959; Demerec & Hartman, 1959). Nevertheless, the availability of DNA suitable for direct analysis is so far limited to that of bacteriophage, in which the DNA still contains many genetic loci.

According to present views genetic recombination, considered as a molecular process, in most diploid forms is limited to exchanges between whole chromosomes or parts of chromosomes which result in the recombination in the progeny of entire blocks of genetic material coming from both parents. Although this material is indeed essentially DNA, the process is hardly one of recombination at a molecular level.

So far, only of bacteria can it be said that molecular recombination takes place. In the process of genetic transformation in bacteria, to be discussed by Hotchkiss, recombination takes place via the transfer and incorporation of specific molecules of DNA. In the phenomenon of transduction, discovered by Zinder & Lederberg (1952), units of DNA are transferred via a bacterial virus vector.

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The most effective selection pressures, as involved in evolution, are of course exerted on the phenotype of the organism. Accordingly, we will now briefly consider some of the various ways in which the genetic material, the genotype, can control the phenotype. Theoretically, as illustrated in the most direct studies on genic control of enzyme production, the base-sequences of the gene determine the amino acid sequence of proteins and hence their specific enzymatic processes. Gene mutation may result in loss of specific protein production, in the production of non-enzymatically active but serologically related protein, or in the production of modified enzyme proteins with altered substrate affinity or altered physical or biochemical properties such as thermal stability or changed sensitivity to organic or inorganic molecules (Yanofsky & St. Lawrence, 1960; Beadle, 1960). However, it seems that synthesis of enzymatically active protein is also under the control of genes not detectably affecting the quality of enzyme produced, but rather modifying the time and/or rate of synthesis. Many genes defined genetically as "modifiers" or "suppressors" may be of this type. Such genes, presumably affecting the activity of the protein synthesizing system, may be very important to the functioning of an organism, and may therefore be subject to selective pressures. As an example, the "thi-low" gene in *Neurospora* (Eberhart & Tatum, 1959) lowers the rate of thiamine synthesis, but can only be detected under particular stress conditions. Under these conditions, which include either genetic or environmental factors, the mutant form of such a gene should be strongly selected against.

Non-allelic suppressor genes may affect either the quantity or quality of enzyme formed (Yanofsky & St. Lawrence, 1960; Mohler & Suskind, 1960) and in part may be responsible for the production of "iso-enzymes" (Kaplan *et al.*, 1960) in different tissues of an organism. That is, different suppressor genes may be activated in these various tissues. Alternatively iso-enzyme production may be under control of different non-allelic primary genes, perhaps evolutionarily related, but differentially activated in different organs and tissues. It would be most important to have some definitive evidence as to the genetic control of these enzymes.

In any consideration of gene-action it must be emphasized that a gene is not like an inorganic or even an organic catalyst, in that it cannot function independently of the living cell with its entire genome. This concept is actually the essence of Goldschmidt's views of the gene. An essential correlate of this concept is that in a living system gene expression is regulated and controlled both by other genes and by the internal and external environment of the cell, as so beautifully outlined by Goldschmidt in connection with developmental processes.

In terms of the gene : enzyme relationship, a number of regulatory mechanisms are now known, especially in micro-organisms, which may serve as models for the control of enzyme production or activity within the limits set by the genome. Such control is presumably concerned with gene expression during developmental processes. Enzyme synthesis may be induced by a critical concentration of substrate molecules, either exogenous or endogenous. If exogenous, the activity of transport systems or "permeases" is important in initiation of enzyme synthesis. Endogenous substrate molecules may accumulate under certain conditions, and trigger the synthesis of an entire sequence of enzymes, as shown for the metabolism of dehydroshikimic acid in *Neurospora* (Gross, 1959). The potentiality for synthesis of inducible enzymes is also under gene control, and mutation has been shown to convert an inducible system either into a non-inducible one or into a constitutive one.

Regulation of gene activity by product feed-back repression of enzyme synthesis has been established for a number of biosynthetic pathways (Vogel, 1957). Feed-back inhibition of enzyme activity has also been well established (Magasanik *et al.*, 1959; Pardee *et al.*, 1959). That repression and induction of enzyme biosynthesis represent opposite sides of the same coin has been suggested, and in bacteria it has been shown that enzyme synthesis controlled by a number of genes involved in a given biosynthetic sequence, and genetically closely linked, may be simultaneously repressed through the action of an end-product of the sequence on still another gene, the "operon", located nearby on the chromosome (Jacob & Monod, 1959).

It will be difficult, if not impossible, to interpret these complex, inter-related phenomena in molecular terms until more is known than at present regarding the entire chain of events and mechanisms leading from the gene to protein synthesis. It seems clear, however, that RNA is vitally involved, both in the transfer of information from DNA in the nucleus to the protein synthesizing ribosomes in the cytoplasm, and in the biosynthesis of the protein. A close relationship of the base sequences of DNA to those of RNA has been suggested by the formation of mixed double helices (Rich, 1960; Hall & Spiegelman, 1961). Such a relationship is obviously of great importance both in the expression of DNA determined characters, and in the replication of genetic virus RNA. With the availability of an RNA bacterial virus as recently described by Loeb & Zinder (1961), these processes can be much more readily attacked. It will also be of great interest to determine whether such RNA viruses can transduce bacteria.

It seems obvious that in evolution selective pressures must operate against the phenotype at all levels from DNA, through RNA, to protein synthesizing systems,

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and at the level of protein function and regulation, but that the variety of phenotypic characters on which selection operates is provided by mutational changes in nucleic acid structure, and fixation in a species of a selected phenotype means selection of a particular nucleic acid molecule. It also seems evident that in many instances it will be difficult if not impossible to determine the level of operation of selection in the complexity of organization of an organism living in a complex environment. Nevertheless, it may be instructive to point some possible examples of selection at different levels.

At the DNA level, some mutations may result in base pair sequences which, through effects on secondary and tertiary molecular structure, render the nucleic acid incapable of normal replication, and hence are eliminated. Others may be replicated, but represent a "non-sense" sequence such that the corresponding protein cannot be synthesized. If this protein has a vital function, and the products of its enzymic action cannot be supplied, this would be a lethal mutation, and would be eliminated in homozygous state selective pressure at the protein level. This would also be true of mutations which permit protein synthesis, but which lead to the production of non-functional enzymes. If the change leads to an enzyme with altered properties, it may be retained for some time even in the homozygous state, but the organism may not survive under rigorous conditions. Such a change could, however, be advantageous to the organism under certain other conditions, and thus play a significant part in evolution. Changes in genes not affecting the enzyme itself, but concerned with the rate of synthesis or its regulation via repressor systems, would be similarly subject to selective pressures.

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Internal selection may be operating at the developmental level against mutations which result in faulty development of some vital tissue or organ in the organism, which cannot survive to reproduce, or may reproduce inefficiently. Similarly, mutant genes which result in lessened survival or functioning of the whole organism will be subject to environmental selective forces. Finally, mutant genes may affect the ecological relationships of the organism and, depending on the environment, may be selected for or against in nature.

The question of the origin of life, and the role of nucleic acid in this phenomenon, has been discussed extensively (Keosian, 1960) since the appearance of the stimulating treatise of Oparin, and recently a great deal of work has been directed toward the *in vitro* synthesis of complex molecules under conditions compatible with life. In this session Lwoff will consider one of the major problems of biology related to the origin and evolution of living things, the origin of viruses.

Rather than discussing or speculating further on the origin of life, it seems best here to consider some aspects of the role of genetic material in the evolution of biosynthetic reactions and enzymes concerned therein. One of the major problems posed by Oparin's thesis that the first organisms were dependent on their environment, the "primeval soup", for the complex molecules necessary for their growth and reproduction, is the evolutionary process by which the complex pathways of synthesis of these molecules from simple compounds were acquired, with all of the requisite enzymes, each specified by its own gene. A number of years ago Horowitz

(1945) proposed an ingenious solution to this problem, consistent with theories of mutation and selection. He suggested that the acquisition of new steps in a biosynthetic sequence went from the end-product backwards one step at a time, with the acquisition of the enzymatic ability to form each product and intermediate from its immediate precursor available in the organic soup. Stabilization of this ability through its fixation in the genetic nucleic acid followed, with a selective advantage for the organism. Exhaustion of the first precursor would cause a repetition of the process until the entire gamut of enzymes needed for the complete synthesis of the needed molecule had been acquired and fixed in the genome.

Inherent in this, or any other hypothesis dealing with the acquisition of any new functions, is the assumption that each new enzymatic function can survive only if new genetic material is available to stabilize and perpetuate the new function. The most reasonable source of new genetic material would seem to be the duplication of an existing gene as a consequence of a process analogous to unequal crossing-over. The duplicate gene could then undergo mutation until, perhaps only by chance, it made a protein which carried out the needed enzymatic function. The selective advantage of this mutation would ensure its survival, and that of subsequent mutations leading to improved enzymatic efficiency.

New gene functions might, in an analogous fashion, have been acquired as the result of mutation of genes which no longer had needed functions as the consequence of mutations early in a biosynthetic sequence which was no longer essential to the organism. These "liberated" genes might mutate and take over a new series of enzymatic steps leading from the accumulated precursor before the earlier block. In this way an entirely new sequence of biochemical steps might be developed, either parallel with or divergent from the original sequence. If we term the Horowitz hypothesis "backward evolution by gain", the last discussed might be called "forward evolution by loss". This forward evolution might involve first the inducted biosynthesis of a series of enzymes in response to the accumulated endogenous compound, as for enzymes involved in dehydroshikimic acid metabolism by *Neurospora* (Gross, 1959), with subsequent mutation of the genes concerned to constitutive production of the enzymes, as shown for certain bacterial enzyme systems.

One possible way in which certain biosynthetic processes and enzymes might become non-essential, thus freeing the related genes to develop new functions, is for mutation to limit an organism to a more restricted biological niche, as with the nutritionally fastidious obligate parasites. This might be viewed as roughly analogous to a geographical isolating mechanism in evolution. As in these processes, a number of unrelated mutant genes might be isolated by chance in the population now restricted to the new biological niche, so that the phenomenon of genetic drift, in addition to simple operation of selective forces, might be involved in further biochemical evolution.

The hypothesis that evolution of genetic material via gene duplication and mutation may have taken place is consistent with several lines of evidence. The similarity in amino acid sequences of the α and β chains of hemoglobin (Braunitzer

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et al., 1960) suggests the evolution of the two non-allelic genes controlling the two chains from a common ancestral gene. The control of the γ-chain in foetal hemoglobin by a gene not allelic with that controlling the adult β-chain likewise may have resulted from the suggested type of evolution of genetic material. The close linkage of genes controlling sequential steps in a biosynthesis (Demerec & Hartman, 1956; Yanofsky & Lennox, 1959; Gross & Fein, 1960) likewise may be taken to suggest that these genes have evolved from a single common ancestral gene, perhaps during the course of Horowitz's backward evolution. The selective pressure needed to keep them in this close genetic relationship could have been that in close proximity their activity could be controlled by a single repressor system, as suggested by Jacob (1958).

In conclusion, I want to emphasize that rapid and illuminating as advances in the molecular aspects of biochemical genetics have been in the past decade, more questions and problems have been raised than answered. Central, and most challenging, is that of the code relating nucleic acid and protein structures. Solution of this problem will depend on continued progress in the determination of the precise structures of the molecules concerned, and on developing knowledge of protein biosynthesis. More intriguing, from the evolutionary aspect, is the further question of how and when in evolution the relationship of nucleic acid and protein first arose. Did this event occur more than once, or was it in fact synonymous with the origin of life? If so, how did the countless genes now present in living organisms develop from the primeval prototype gene? Perhaps the other speakers in this symposium will provide leads towards the solution of these and related questions—I can only pose some of them. In so doing, however, I feel that I have the right as a biochemist, and the responsibility as a geneticist and biologist, to warn that living organisms are much more than mixtures of enzymes and nucleic acid molecules, and that important and basically simple as the molecular aspects of biochemical genetics may seem, Nature may still have many unpredictable surprises in store for us.

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THE STRUCTURE OF GLOBULAR PROTEINS

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THE study of the structure of globular proteins by X-ray methods began in the late 1930's when the first X-ray photographs of protein crystals were obtained by Bernal, Crowfoot, Fankuchen and Perutz. Proteins are so much more complex than the molecules which were within the scope of X-ray methods at this time and for many years afterwards, that little progress was made in terms of results, though much in terms of techniques, for about 15 years. The decisive advance came in 1953, when Perutz showed (Green *et al.*, 1954) that it was possible to prepare heavy-atom derivatives of haemoglobin which crystallized isomorphously with the unsubstituted protein and whose crystals gave appreciably different diffraction patterns; he was then able to use the so-called method of isomorphous replacement which in principle enables an unambiguous structure determination to be carried out. During more recent years a number of crystalline proteins has been studied intensively with the object of applying this method, especially haemoglobin, myoglobin, insulin, ribonuclease and lysozyme. Always the difficulty has been to obtain suitable isomorphous heavy-atom derivatives; only one chemically rational method—the attachment of mercury or silver atoms to free sulphydryl groups, as originally used by Perutz—is known even today, and this is more often than not inapplicable since most of the proteins suitable for analysis on other grounds (e.g. low molecular weight, ease of crystallization) do not contain free sulphydryl groups. For the most part it has been necessary to search *ad hoc* for methods of attaching heavy atoms, those used successfully for one crystalline protein generally not being applicable to others. Only in haemoglobin and myoglobin has the isomorphous replacement method so far been applied with complete success. Perutz's early results with haemoglobin took the form of a projection of a row of haemoglobin molecules onto a plane—they were the first pictures of a protein molecule which were demonstrably correct, but the amount of structural information which could be derived from what was in effect a silhouette of a very complicated molecule was small. It was clear that if the method was to be exploited it was necessary to extend it into the third dimension, and this was first achieved in 1957 in the analysis of the simpler protein myoglobin (Bodo *et al.*, 1959).

The first stage of the X-ray analysis of myoglobin resulted in a three-dimensional representation of the molecule at a resolution of 6 Å, insufficient to reveal the side-chains or even the configuration of the polypeptide chain itself, but which did give a picture of the general arrangement of the polypeptide chain and of the haem group—in other words of the tertiary structure of the molecule. This tertiary structure proved to be unexpectedly irregular and complex: clearly the simple

geometrical arrangements proposed in many earlier theories of protein structure could not be correct. More recently the resolution of the analysis has been increased to 2 Å (Kendrew *et al.*, 1960). Although neighbouring covalently bonded atoms are still not distinctly resolved, it is now possible to separate atoms which are hydrogen-bonded or in Van der Waals contact, with the result that the atomic arrangement of most of the molecule can be inferred. At this resolution the appearance of the haem group with its central iron atom corresponds closely with theoretical expectation, thus providing a valuable check on the correctness of the analysis, and it can be seen that the iron atom is attached to a neighbouring polypeptide chain by means of a group which is almost certainly the imidazole ring of a histidine residue. Most of the polypeptide chain consists of a series of segments of right-handed α-helix, with parameters closely corresponding to those proposed by Pauling *et al.*, 1951. This was the first time that the α-helix had been directly seen in a protein molecule; the results confirmed earlier plausible evidence that this configuration was important in the structure of globular proteins as well as in those of many fibrous proteins. It turns out that the myoglobin molecule consists of eight segments of α-helix joined by irregular regions of varying length: the helical segments comprise 75 per cent of the amino-acid residues, in agreement with estimates made on the basis of optical rotation and deuterium exchange studies.

One reason for the choice of 2 Å as the resolution of the second stage of the myoglobin analysis was that the secondary structure (or polypeptide chain configuration) should be clearly revealed at this resolution, as indeed proved to be the case; it was not anticipated that side-chains would be identifiable, so that progress could be made towards a direct determination of the amino-acid sequence by X-ray methods alone. In the event expectation was much exceeded, for it was possible to see the individual side-chains as dense regions emerging from the helical main chain at intervals corresponding to the parameters of the α-helix, and a close examination of these regions often made it possible to identify side-chains with certainty; in other cases some ambiguity remained but the choice of side-chain could be reduced to two or three. At the present resolution about one-third of the side-chains can be identified with certainty, and another third with fairly high probability (Kendrew *et al.*). It now becomes possible to correlate these X-ray results with the preliminary data obtained by Edmundson who is engaged in working out the amino-acid sequence of myoglobin by chemical methods (Green *et al.*, 1954). He has broken down the molecule into short peptides by means of tryptic digestion, and he has determined the composition, and in a few cases the internal sequence, of these peptides. By comparing his results with our own it has proved possible to place almost all the tryptic peptides along the polypeptide chain, and the order of peptides thus ascribed corresponds with the order suggested in a few cases by Edmundson on the basis of preliminary work using chymotryptic digestion. Although some discrepancies remain and although the amino-acid sequence has not yet been completely determined, its main features are now beyond doubt. We are at present engaged in an attempt to increase the resolution of the X-ray results still further, and we hope that the remaining ambiguities will then be removed.

Meanwhile Perutz (1960) and his collaborators have obtained a three-dimensional representation of the haemoglobin molecule at a resolution of 5·5 Å. Haemoglobin is known to be made up of four sub-units, each consisting of a single polypeptide chain and an associated haem group. Larger protein molecules generally consist of several sub-units, whose spatial arrangement is termed the quaternary structure; Perutz's results show that in haemoglobin the quaternary structure is of a very simple and symmetrical kind, the four sub-units being arranged at the vertices of a nearly regular tetrahedron. The most astonishing result of the analysis, however, is that the tertiary structure of each of the four sub-units very closely resembles that of myoglobin; by implication it follows that the secondary structure must also be very similar. This remarkable finding indicates that the irregular arrangement of the polypeptide chain now found in two distinct proteins, derived from very different species, namely horse and sperm whale, has some general significance which we cannot at present understand. A further extension of the range of this structure has been provided by the work of Scouloudi (1960), who has shown that the molecule of seal myoglobin very closely resembles that of horse myoglobin.

The structures of virus particles lie outside the scope of the present paper; it is, however, relevant to note that in these still more complex molecules protein subunits are also arranged in a highly regular manner. For example, in tobacco mosaic virus the 2000 identical protein sub-units, each as large as a myoglobin molecule, are arranged in the form of a helix; in the spherical viruses the sub-units are disposed symmetrically on the surface of regular or semi-regular polyhedra.

Thus we are now beginning to get a first glimpse at the general nature of protein structure at all levels of complexity. Hitherto the chief difficulty in applying to biological problems the results of the exhaustive physico-chemical and chemical studies of proteins carried out in many laboratories during the last half-century has been our lack of knowledge of the three-dimensional arrangement of the atoms of which protein molecules are composed. Such knowledge is an essential complement to an understanding of the chemical interconnections, because it is the spatial relations between the side-chains which determine the chemical behaviour and biological specificity of the protein molecule as a whole, and these relations cannot be determined, except in a fragmentary manner, by purely chemical techniques. The rate of progress on all these fronts is now so great that we are undoubtedly on the eve of a major advance in our understanding of one of the central problems of biology—the relation between the structure of the molecules making up living cells and their biological functioning.

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PHOTOSYNTHETIC PHOSPHORYLATION AND A UNIFIED CONCEPT OF PHOTOSYNTHESIS

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PHOTOSYNTHESIS in plants and photosynthetic bacteria is defined here as the synthesis of cellular substances at the expense of chemical energy, formed by photochemical reactions. This definition includes, but is not limited to, the assimilation of CO_2. Carbon assimilation in photosynthetic cells is fundamentally the same dark process which occurs also in non-photosynthetic cells as, for example, in *Thiobacillus denitrificans*. In both photosynthetic and nonphotosynthetic cells, the dark reactions of carbon assimilation are driven by ATP and reduced pyridine nucleotide (PNH_2), but only in photosynthetic cells are these two substances formed at the expense of light energy. The first stable, chemically defined products of photosynthesis are not intermediates of CO_2 assimilation but are ATP and PNH_2. The two photochemical reactions that form ATP and PNH_2 in plants and photosynthetic bacteria are cyclic and noncyclic photophosphorylation. Cyclic photophosphorylation is viewed as a basically anaerobic process that produces only ATP. Noncyclic photophosphorylation produces ATP, PNH_2 and O_2 in chloroplasts but in photosynthetic bacteria it produces ATP and PNH_2 without O_2. By replacing water (OH^-) with another electron donor, noncyclic photophosphorylation in chloroplasts has been experimentally converted to the bacterial type in which ATP and PNH_2 are formed without oxygen evolution.

INTRODUCTION

Photosynthetic phosphorylation is a term coined in 1954 to describe a light-induced ATP formation by isolated chloroplasts, in accordance with equation 1:

$$ADP + H_3PO_4 \xrightarrow{light} ATP \qquad (1)$$

Reaction 1, in which the sole product is ATP, was subsequently designated cyclic photophosphorylation (Arnon *et al.*, 1958; Arnon, 1959), to distinguish it from a second photophosphorylation reaction by isolated chloroplasts (equation (2)) which we found a few years later (Arnon *et al.*, 1958) and named noncyclic photophosphorylation (Arnon, 1959).

$$2TPN + 2H_2O + 2ADP + 2H_3PO_4 \xrightarrow{light} 2TPNH_2 + O_2 + 2ATP \qquad (2)$$

* The recent phases of the work from the author's laboratory described herein have been carried out in collaboration, particularized in the bibliography, with M. Losada, M. Nozaki, K. Tagawa, F. R. Whatley, D. O. Hall, H. Y. Tsujimoto and A. A. Horton. The work was aided by grants from the U.S. Public Health Service and the Office of Naval Research.

In cyclic photophosphorylation all of the biochemically effective light energy is used for ATP formation. In noncyclic photophosphorylation only a portion of the biochemically effective light energy is used for the formation of ATP; the remainder is used for the formation of a reductant, $TPNH_2$ and the evolution of oxygen. A nonphysiological variant of noncyclic photophosphorylation (Reaction 2) is Reaction 3, in which TPN is replaced by ferricyanide (Arnon *et al.*, 1958) (represented here by Fe^{3+}).

$$4Fe^{3+} + 2H_2O + 2ADP + 2H_3PO_4 \rightarrow 4Fe^{2+} + O_2 + 2ATP + 4H^+ \qquad (3)$$

The discovery of light-induced ATP formation by isolated chloroplasts was confirmed and extended in other laboratories, notably those of Jagendorf (Avron & Jagendorf, 1957; Avron *et al.*, 1958; Jagendorf, 1958; Jagendorf & Avron, 1958, 1959), Wessels (1957, 1958), Vennesland (Chow & Vennesland, 1957; Krogmann & Vennesland, 1959; Nakamoto *et al.*, 1959), and Hill & Walker (1959). Most of the work has been done with spinach chloroplasts but recently Whatley *et al.* (1960) have also demonstrated cyclic and noncyclic photophosphorylation in chloroplasts isolated from several other species of plants.

Chloroplasts were once widely believed (Sachs, 1887; Pfeffer, 1900) to be the site of complete photosynthesis, that is, of oxygen evolution coupled with carbon dioxide assimilation. This view was not supported by critical experimental evidence and was later abandoned (Brown & Frank, 1948; Benson & Calvin, 1950; Rabinowitch, 1952; Lumry *et al.*, 1954) when Hill (1951) found in 1937 that isolated chloroplasts produce oxygen in light but cannot assimilate CO_2. Thus Rabinowitch (1953) wrote that "the photosynthetic process, like certain other groups of reactions in living cells, seems to be bound to the structure of the cell; it cannot be repeated outside that structure".

The capacity of isolated chloroplasts for complete photosynthesis was established (Arnon *et al.*, 1954; Arnon, 1955; Allen *et al.*, 1955) when, coincident with the discovery of photosynthetic phosphorylation (Arnon *et al.*, 1954), it was observed that chloroplasts were able not only to evolve oxygen but also to convert $^{14}CO_2$ to starch and sugar, at physiological temperatures and with no energy supply except visible light. The conversion of $^{14}CO_2$ by isolated chloroplasts to sugars and starch was confirmed and extended in other laboratories* (Gibbs & Cynkin, 1958; Tolbert, 1958; Gibbs & Calo, 1959; Smillie & Krotkov, 1959; Smillie & Fuller, 1959; Veda, 1949; Irmak, 1955; Thomas *et al.*, 1957).

By fractionating isolated chloroplasts, it was later demonstrated (Trebst *et al.*, 1958) that their overall process of photosynthesis can be experimentally separated into a light and a dark phase. The dark phase was found to consist of the same

* Other accounts of the discovery of CO_2 assimilation and photosynthetic phosphorylation by isolated chloroplasts sometimes are found in the literature. Thus Bassham & Calvin (1956, p. 31) ascribed the discovery of CO_2 assimilation by isolated chloroplasts to Boychenko & Baranov (1954). Later, Calvin (1959, p. 152) ascribed the discovery of both CO_2 assimilation and ATP synthesis by isolated chloroplasts to his own laboratory in 1959.

enzymatic reactions (Trebst *et al.*, 1958; Losada *et al.*, 1960; Trebst *et al.*, 1960) that convert CO_2 into carbohydrate in intact green cells (Calvin, 1955; Weissbach *et al.*, 1956; Jakoby *et al.*, 1956; Racker, 1957). These dark reactions are driven by ATP and reduced TPN (jointly named assimilatory power (Arnon *et al.*, 1958)) that are formed during the light phase by cyclic and non-cyclic photophosphorylation.

An important link in the chain of evidence connecting the light phase of photosynthesis with the dark assimilation of CO_2 in chloroplasts was provided by experiments showing that both cyclic and noncyclic photophosphorylation are essential (Trebst *et al.*, 1959). Noncyclic photophosphorylation (equation (2)) provides the three products of the light phase of photosynthesis, O_2, $TPNH_2$ and ATP. Cyclic photophosphorylation (equation (1)) supplies only ATP and the participation of this reaction in CO_2 assimilation would be needed only if the ATP formed in Reaction 2 alone were insufficient for CO_2 assimilation to the level of carbohydrate. This was found to be the case; CO_2 assimilation to the level of carbohydrates depended on a properly balanced participation of both cyclic and noncyclic photophosphorylation (Trebst *et al.*, 1959).

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It thus becomes clear (Trebst *et al.*, 1958; Loisada *et al.*, 1960) that the dark reactions of CO_2 assimilation in chloroplasts are removed from the domain of photosynthetic reactions proper in which light is converted into chemical energy. There was a firm experimental basis for concluding that the energy conversion process in photosynthesis manifests itself as cyclic and noncyclic photophosphorylation, and that their products, $TPNH_2$ and ATP, suffice for converting CO_2 into carbohydrates (Trebst *et al.*, 1958; Trebst *et al.*, 1959; Losada *et al.*, 1960; Trebst *et al.*, 1960). The experimental facts in photosynthesis by isolated chloroplasts argue against postulations of a special, photochemically produced "reducing power" that is capable of direct reduction of CO_2 to the level of carbohydrate by some unknown reaction peculiar to photosynthesis (see, for example, Strehler (1953) and Gaffron (1954)).

This article will discuss some of the special features of cyclic and noncyclic photophosphorylation that lead to a unified concept of photosynthesis in plants and bacteria. Although this concept has already passed the test of predicting certain experimental findings in and beyond conventional photosynthesis (Arnon *et al.*, 1960) it still remains tentative in some particulars and is presented here in a spirit that disavows a "premature attachment to hypothesis" (Priestley, 1777).

The treatment of literature will be selective rather than comprehensive. More exhaustive reviews of recent work are available elsewhere (Jagendorf, 1958; Baltscheffsky, 1960; Arnon, 1960; Arnon, 1961a, b).

UNIQUE FEATURES OF CYCLIC PHOTOPHOSPHORYLATION

Photosynthetic phosphorylation has been observed under experimental conditions that exclude CO_2 assimilation by chloroplasts. No CO_2 was supplied to the reaction mixture and the reaction vessels contained KOH in the center well. The possibility cannot be completely excluded, however, that even under these

conditions, residual CO_2 may have had some catalytic function in the photochemical reactions, as was recently proposed by Warburg *et al.* (1959) for the photoevolution of oxygen by chloroplasts. What can be excluded is that substrate amounts of carbon compound(s) are first synthesized in light and are then used as electron donors for the formation of ATP.

Of the two photophosphorylation reactions (equations (1) and (2)), the cyclic type (equation (1)) appeared the more basic from the standpoint of mechanisms of energy transformation in photosynthesis. The sole product of cyclic photophosphorylation is ATP. Cyclic photophosphorylation thus separates the key event of photosynthesis, the conversion of light into chemical energy, from the two cornerstones of photosynthesis in green plants: the reduction of CO_2 and the evolution of oxygen. The sole product of cyclic photophosphorylation, ATP, is a highly negotiable physiological "energy currency" that can be used for a multitude of endergonic reactions within the cell including, but not limited to, CO_2 assimilation.

The significance of cyclic photophosphorylation as a basic energy conversion process in photosynthesis was greatly increased when it became clear that it is not limited to chloroplasts but is apparently found in all photosynthetic cells. Soon after the discovery of photosynthetic phosphorylation in chloroplasts, Frenkel (Avron *et al.*, 1958) demonstrated photosynthetic phosphorylation in cell-free preparations of a purple, nonsulfur photosynthetic bacterium, *Rhodospirillum rubrum*, a finding confirmed by Geller (1957). Williams (1956) found photosynthetic phosphorylation in the anaerobic sulfur bacteria, *Chromatium* and *Chlorobium*; photophosphorylation by *Chromatium* particles was further investigated by Kamen & Newton (1957) and Anderson & Fuller (1958). Thomas & Haans (1955) and Petrack (1959) found cyclic photophosphorylation in algal preparations.

Photosynthetic phosphorylation in bacteria seemed to differ at first from that in chloroplasts in its dependence on a chemical substrate. Frenkel's cell-free preparations of *R. rubrum* became substrate-dependent after washing; the rate of phosphorylation was doubled on adding α-ketoglutarate (Frenkel, 1954). But in subsequent experiments Frenkel (1956) ruled out the dependence on an added chemical substrate and once this fundamental point was clarified, the equivalence of cyclic photophosphorylation in chloroplasts and bacterial particles seemed probable (Geller, 1957).

The unique features of cyclic photophosphorylation are that ATP is formed without the consumption of an exogenous electron donor and an electron acceptor. In respiration ATP is formed at the expense of free energy released during the flow of electrons from the respiratory substrate to molecular oxygen, both of which are consumed in the process. Cyclic photophosphorylation consumes neither respiratory substrate nor molecular oxygen. The free energy required for the synthesis of pyrophosphate bonds of ATP is supplied solely by absorbed light.

Since cyclic photophosphorylation emerged as a unique process, common to all photosynthetic organisms, it seemed reasonable to expect that the elucidation of its mechanism would prove important to the understanding of the overall energy

conversion process in photosynthesis. Its mechanism may be divided into three phases: (a) the light-induced generation of endogenous electron donor and endogenous electron acceptor, (b) electron transport from the donor to the acceptor via a photosynthetic electron transport chain, and (c) phosphorylation reactions coupled to electron transport. Phases (b) and (c) are analogous and possibly identical in some respects with their counterparts in oxidative phosphorylation, whereas phase (a) is peculiar to cyclic photophosphorylation, and will therefore be stressed here.

THE ELECTRON FLOW MECHANISM OF CYCLIC PHOTOPHOSPHORYLATION

The first working hypothesis proposed (Arnon *et al.*, 1955) for the mechanism of cyclic photophosphorylation attempted to account for this reaction within the framework of van Niel's hypothesis of photolysis of water. This hypothesis visualized cyclic photophosphorylation as resulting from the simultaneous generation from water of a reducing (H) and an oxidizing moiety (OH) at opposite ends of an electron transport chain. Vitamin K, riboflavin phosphate (FMN), ascorbate and cytochromes were tentatively proposed (Arnon *et al.*, 1955) as members of the electron transport chain which catalyzed a stepwise recombination of the products of photodecomposition of water, thereby making energy available for the formation of pyrophosphate bonds. The role of the first electron or hydrogen acceptor was tentatively assigned to either FMN or, as was suggested by Wessels, to vitamin K. FMN, vitamin K and ascorbate were (in addition to Mg^{2+}) substances known to occur in chloroplasts, and were found to catalyze strongly cyclic photophosphorylation (Arnon *et al.*, 1954; Whatley *et al.*, 1955; Arnon *et al.*, 1955).

We have later abandoned this hypothesis in favor of an electron flow mechanism (Arnon, 1959, 1961b) which agreed better with the more recent experimental findings. The electron flow theory dispenses with photolysis of water as the reaction that gives rise to the electron donor and the electron acceptor. Instead, it envisages that chlorophyll, on absorbing a photon in the primary photochemical act, expels an electron having a high energy potential that it attains at the expense of absorbed light energy. Chlorophyll thus becomes an electron donor. On losing an electron, chlorophyll becomes an electron acceptor, i.e. it becomes electropositive (acquires a "hole") and compensates for this electron deficiency by accepting an electron from cytochrome which, in turn, becomes oxidized. The electron which has been expelled from chlorophyll passes through the photosynthetic electron transport chain, where it finally reaches the oxidized cytochrome. During these electron transport steps, the coupled enzyme systems convert electron energy into the pyrophosphate bond energy of ATP. We envisage the photosynthetic electron transport chain as including cytochromes, and FMN, vitamin K or their related physiological equivalents.

A diagram illustrating this concept is shown in Fig. 1. The electrons that are activated by light and expelled from chlorophyll return to it by a cyclic

path—hence the name cyclic photophosphorylation. Evidence in support of this mechanism is discussed elsewhere (Arnon, 1959, 1961a, b).

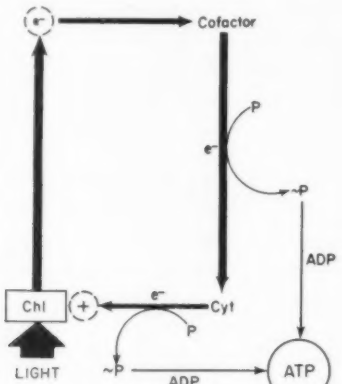

FIG. 1. Scheme for anaerobic cyclic photophosphorylation catalyzed by vitamin K_3 or FMN (cofactor). Details in the text.

Certain nonphysiological agents, such as phenazine methosulfate, have been found to catalyze cyclic photophosphorylation in chromatophores (Geller, 1957; Kamen & Newton, 1957) and chloroplasts (Jagendorf, 1958) and presumably act by providing artificial "shortcuts" (Geller, 1957) for the physiological electron pathway. At high light intensity the rate of cyclic photophosphorylation catalyzed by phenazine methosulfate exceeds that catalyzed by vitamin K or FMN (Arnon, 1961a, b). This has been interpreted (Arnon, 1961a, b) as being the result of phenazine methosulfate by-passing a slow step (Geller, 1957) in the electron transport chain (phenazine methosulfate has been shown by Massey (1959) to catalyze a very rapid electron transfer to cytochrome-c).

At low light intensity the overall rate of cyclic photophosphorylation is limited by the electron flow from excited chlorophyll. Under these conditions the rate of photophosphorylation catalyzed by vitamin K or FMN was about twice that catalyzed by phenazine methosulfate, suggesting that with cofactors that are in themselves, or can substitute for, physiological catalysts, more phosphorylating sites were operative (Arnon, 1961a, b) (cf. Fig. 1 and Fig. 2).

MECHANISMS OF NONCYCLIC PHOTOPHOSPHORYLATION IN CHLOROPLASTS

As already stated, in noncyclic photophosphorylation by chloroplasts the formation of ATP is accompanied by oxygen evolution and the reduction of an added electron acceptor, TPN (equation (2)). We have suggested (Arnon, 1959, 1961a, b) that in this process the electrons expelled by chlorophyll are accepted by TPN, which simultaneously receives H^+ from water to form reduced TPN.

The electrons lost by chlorophyll are restored, via cytochrome, by electrons from OH^-—a reaction that is envisaged as resulting in ATP formation and concomitant oxygen evolution. In this proposed mechanism of noncyclic photophosphorylation,

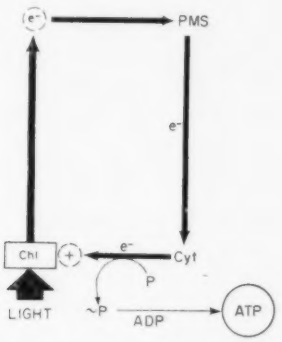

FIG. 2. Scheme for anaerobic cyclic photophosphorylation catalyzed by phenazine methosulfate (PMS). Details in the text.

oxygen is an excreted by-product incidental to the use of water (OH^-) as the electron donor.

It was already known that isolated chloroplasts or even solutions of chlorophyll could reduce TPN with electron donors other than water, but this photoreduction was not previously shown to be coupled with ATP formation. Vernon & Zaugg (1960), extending the earlier work of Vernon & Kamen (1954), have shown that chloroplasts which have lost, through aging or by poisoning, the capacity to photoevolve oxygen are nevertheless able to photoreduce TPN with electrons donated by a reduced dye, 2,6-dichlorophenol indophenol. (Operationally, the dye is supplied in catalytic amounts and kept in a reduced form by substrate amounts of added ascorbate.) Krasnovsky (1960) had found earlier that chlorophyll solutions in pyridine can bring about a photochemical electron transfer from ascorbate or other electron donors to pyridine nucleotides or other electron acceptors.

In recent experiments Losada *et al.* (1961) have separated noncyclic photophosphorylation in chloroplasts (equation (2)) into two distinct photochemical reactions, (a) a photo-oxidation of water (OH^-) that yields oxygen (equation (4)) and (b) a photophosphorylation reaction proper in which the photoreduction of triphosphopyridine nucleotide is coupled with the formation of adenosine triphosphate (equation (5)). The two reactions have been separated by using reduced (A^-) and oxidized (A) indophenol dyes as summarized below:

$$H_2O + 2A \xrightarrow{light} 1/2O_2 + 2A^- + 2H^+ \qquad (4)$$

$$2A^- + TPN + 2H^+ + ADP + H_3PO_4 \xrightarrow{light} TPNH_2 + ATP + 2A \qquad (5)$$

$$\text{Sum: } TPN + ADP + H_3PO_4 + H_2O \xrightarrow{light} ATP + TPNH_2 + 1/2O_2 \qquad (2)$$

NONCYCLIC PHOTOPHOSPHORYLATION WITHOUT OXYGEN EVOLUTION

Table 1 shows that when catalytic amounts of reduced 2,6-dichlorophenol indophenol replaced water (OH^-) as the electron donor (Treatment 3), illuminated chloroplasts formed ATP and reduced TPN without evolving oxygen. Similar

TABLE 1—NONCYCLIC PHOTOPHOSPHORYLATION BY CHLOROPLASTS WITH AND WITHOUT OXYGEN EVOLUTION

No.	Addenda	Effective electron donor	Oxygen evolved (μatoms)	Triphosphopyridine nucleotide reduced (μmole)	Adenosine triphosphate formed (μmole)
1.	None	Water	3·0	3·4	2·4
2.	Chlorophenyl dimethyl urea (CMU)	None	0	0·5	0·2
3.	Dye, ascorbate, CMU	Reduced dye (ascorbate)	0	3·2	3·4

Vessel 1 contained in a total volume of 3 ml: washed chloroplast fragments (P1S2), prepared in the absence of chloride, containing 0·5 mg chlorophyll; 0·05 ml purified spinach phosphopyridine nucleotide reductase, and the following in micromoles tris/acetate buffer, pH 8–0, 40; $MgSO_4$, 5; $K_2HP^{32}O_4$, 10; ADP, 10; TPN, 4; and KCl, 10. In treatment 2KCl was omitted and $2 \times 10 = 5$ M *p*-chlorophenyl dimethylurea (CMU) was added. Treatment 3 was the same as Treatment 2 except that 20 μmole ascorbate and 0·2 μmole dye (2,6-dichlorophenol indophenol) were added. The experiment was run for 20 min at 150 (at a light intensity of 2000 ft-cd). Oxygen was measured manometrically and triphosphopyridine nucleotide spectrophotometrically at 340 mμ.

The omission of chloride and the addition of CMU, a powerful inhibitor of oxygen evolution, blocked the use of water as an electron donor in the chloroplast system. Catalytic amounts of 2,6-dichlorophenol indophenol served as an electron carrier between ascorbate and the chloroplast system.

results were obtained when reduced 2,3,6-trichlorophenol indophenol (Krogmann & Jagendorf, 1959) was used instead of 2,6-dichlorophenol indophenol. The participation of water as an electron donor in these reactions was blocked by the addition of *p*-chlorophenyl dimethylurea (Vernon & Zaugg, 1960) to, and the omission of chloride (Bové *et al.*, 1959) from, the reaction mixture. When the participation of water as an electron donor was not blocked (Table 1, Treatment 1), the reduction of TPN and the coupled phosphorylation were accompanied by oxygen evolution. When the participation of water was blocked but no substitute electron donor was supplied (Treatment 2) no significant reaction occurred at all.

The primary photophosphorylation reaction in chloroplasts, when the participation of water as the electron donor is blocked, is shown diagrammatically in Fig. 3.

The electron donor, A^-, represents here the reduced indophenol dye, and the electron acceptor, B, represents TPN (cf. equation (5)). In accordance with previous formulations, we envisage the formation of ATP as occurring during the electron transport between cytochrome and chlorophyll. This photophosphorylation step is envisaged as being common to all pathways of photosynthetic phosphorylation, cyclic and noncyclic, and will henceforth be referred to as the primary photophosphorylation reaction (cf. Figs. 1, 2 and 3).

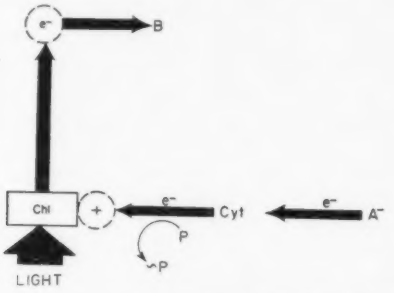

FIG. 3. Scheme for noncyclic photophosphorylation by chloroplasts, without oxygen evolution. The chlorophyll molecule (Chl) becomes excited by the absorption of a quantum of light. The excited chlorophyll donates its high energy electron (e^-) to the electron acceptor (B) and accepts, via the cytochrome system (Cyt), an electron from an external electron donor (A^-). The phosphorylation step is linked with the transfer of the electron from cytochrome to chlorophyll.

PHOTOOXIDATION OF WATER AS A SEPARATE REACTION

In the experiments shown in Table 1 catalytic amounts of phenol indophenol dye mediated the transfer of electrons from ascorbate to TPN. The dye was reduced in the dark by ascorbate and oxidized in the light by TPN. This electron transport was coupled with the formation of ATP (equation (5)).

In parallel experiments (Losada *et al.*, 1961) catalytic amounts to phenol indophenol dye mediated the transfer of electrons from water (OH^-) to ferricyanide. Hence the dye was reduced in the light by water (OH^-) (resulting in an evolution of oxygen) and was oxidized in the dark by ferricyanide. This photooxidation of water was not coupled with the formation of adenosine triphosphate (equation (4)).

Indophenol dyes have long been known (Holt & French, 1948; Krogmann & Jagendorf, 1959) to be reduced photochemically by chloroplasts in a Hill reaction, but unlike such Hill reagents as ferricyanide (Arnon *et al.*, 1958; Avron *et al.*, 1958; Jagendorf, 1958), the photoreduction of 2,3,6-trichlorophenol indophenol, for example, is not linked with photophosphorylation (Krogmann & Vennesland, 1959; Krogmann & Jagendorf, 1959). Krogmann & Jagendorf (1959) drew from this "the inference . . . that reduction of indophenol dye is primarily by means of uncoupled electron transport, possibly by a 'short circuit' of that part of the electron transport system which is coupled to phosphorylation".

We have examined (Losada *et al.*, 1961) the effect of catalytic amounts of indophenol dyes in the variant of noncyclic photosphorylation in chloroplasts where ferricyanide has replaced TPN as the electron acceptor (equation (3)). The results are shown in Table 2. The addition of catalytic amounts of indophenol dye to the ferricyanide system has strongly inhibited the formation of ATP without impairing oxygen evolution (Treatments 2 and 4). The addition of catalytic amounts of indophenol dye has thus converted the noncyclic photophosphorylation reaction with ferricyanide into a more limited reaction, a photooxidation of water that is not accompanied by photophosphorylation.

TABLE 2—SEPARATION OF PHOTOPRODUCTION OF OXYGEN AND PHOTOPHOSPHORYLATION BY CHLOROPLASTS

No.	Treatment	Effective electron acceptor	Oxygen produced (μatoms)	Adenosine triphosphate formed (μmoles)
1.	Ferricyanide	Ferricyanide	4·5	4·7
2.	Dye A + Ferricyanide	Oxidized dye (Ferricyanide)	5·1	1·3
3.	Ferricyanide	Ferricyanide	3·3	3·5
4.	Dye B + Ferricyanide	Oxidized dye (Ferricyanide)	3·5	0·8

The reaction mixture contained, in a final volume of 3 ml, unwashed chloroplast fragments (Pls) containing chlorophyll and the following in micromoles: tris/HCl buffer, pH 8, 100; $MgSO_4$, 5; $K_2HP^{32}O_4$, 10; adenosine diphosphate, 10; potassium ferricyanide, 10. Dye A (0·2 μmole 2,6-dichlorophenol indophenol) or Dye B (0·05 μmole 2,3′,6-trichlorophenol indophenol) was added where indicated. 10% KOH was present in the center well of the manometer vessels. The reaction was run for 12 min at 15°C in the light (2000 ft-cd). The gas phase was nitrogen.

The suppression of APT formation by indophenol dyes in the presence of ferricyanide (Table 2) could not be attributed to inhibiting, uncoupling, "short-circuiting" or by-passing a phosphorylation step in Reaction 3. As shown in Table 3, the addition of indophenol dyes to the noncyclic photo-phosphorylation system with TPN (equation (2)) did not suppress photophosphorylation and has, in fact, slightly increased it. As in the ferricyanide system (Table 2), the addition of the dye to the TPN system did not affect the oxygen evolution.

In the experiments illustrated in Table 2 it was possible to measure the oxygen evolution that accompanies the photoreduction of indophenol dyes because they were supplied in catalytic amounts and were kept in the oxidized state by the

presence of substrate amounts of ferricyanide. A direct measurement of oxygen evolution that accompanies the photoreduction of 2,6-dichlorophenol or 2,3′,6-trichlorophenol indophenol is difficult to make because these dyes, when used in concentrations that are required for manometric measurements of evolved oxygen, absorb too much light. This reduces the effective light intensity to such an extent that chloroplasts cease to evolve oxygen (Holt & French, 1948). Indophenol dyes are therefore usually used as Hill reagents in low concentrations and their photoreduction is measured not manometrically by oxygen evolution but optically by their decolorization (Holt & French, 1948; Jagendorf, 1956).

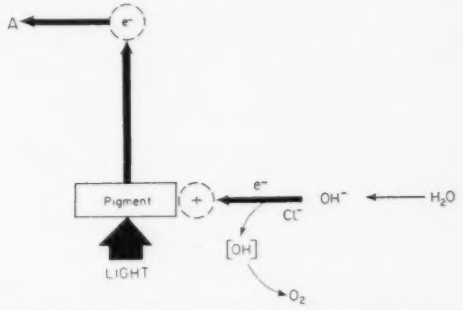

FIG. 4. Scheme for photooxidation of water by chloroplasts. The pigment molecule (possibly chlorophyll-b, see text) becomes excited by the absorption of a quantum of light. The excited pigment donates its high energy electron (e^-) to an intermediate electron acceptor A and accepts a replacement electron from OH^-. The oxidation product (OH) of OH^- is the precursor of oxygen gas (compare the anode reaction in the electrolysis of water).

The photooxidation of water is diagrammatically represented in Fig. 4. The reaction requires chloride (Whatley *et al.*, 1961). We regard the photooxidation of water by chloroplasts as an auxiliary reaction to supply electrons at an elevated reducing potential for a second photochemical reaction, in which the primary photophosphorylation reaction is coupled with the reduction of pyridine nucleotide (Fig. 3).

It appears that noncyclic photophosphorylation in green plants is the sum of two component photochemical reactions: (a) the auxiliary reaction for photooxidation of water (Fig. 4) and (b) the primary photophosphorylation reaction (Fig. 3) coupled with pyridine nucleotide reduction.

Figure 5 depicts the combined scheme for noncyclic photophosphorylation in green plants that we now envisage. The intermediate A in Fig. 5 is both the electron acceptor for the first, and the electron donor for the second light reaction (cf. Figs. 3 and 4). We have used indophenol dyes as an experimental device to separate the two photochemical reactions. The natural intermediates with which the dyes have interacted have not been identified. They may include cytochromes or a quinone.

Under physiological conditions, when the electron acceptor B (Fig. 5) is TPN, noncyclic photophosphorylation in chloroplasts (equation (2)) produces ATP and a strong reductant, $TPNH_2$, having at pH 7, a normal redox potential $E'_0 = -0{\cdot}324$ V. Under nonphysiological conditions, when B is not TPN but a

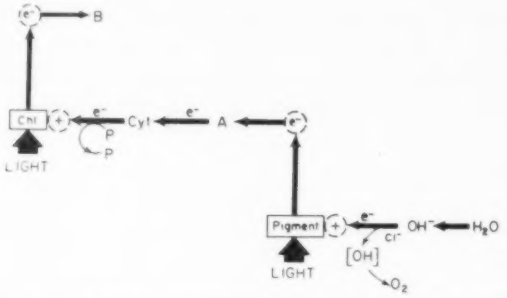

FIG. 5. Scheme for noncyclic photophosphorylation coupled with photo-oxidation of water by chloroplasts. In the first light reaction, the pigment molecule becomes excited by the absorption of a quantum of light. The excited pigment donates its high energy electron (e^-) to an intermediate electron acceptor (A) and accepts an electron from a hydroxyl ion. The oxidation production (OH) of the hydroxyl ion is the precursor of molecular oxygen. In the second light reaction, the chlorophyll molecule (Chl), excited by the absorption of a quantum of light, donates its high energy electron (e^-) to the terminal electron acceptor (B) and accepts, via the cytochrome system (Cyt), an electron from the reduced intermediate (A^-) formed in the first light reaction. The phosphorylation is linked with the transfer of the electron from cytochrome to chlorophyll. Under physiological conditions, TPN serves as the electron acceptor B.

more oxidized electron donor such as ferricyanide ($E'_0 = 0{\cdot}43$ V), a considerable amount of absorbed light energy (equivalent to a redox potential of 754 mV) is lost. ATP (also oxygen) is produced in the same ratio to the number of electrons transferred as with TPN, but the reductant formed is no longer $TPNH_2$ but ferrocyanide.

According to the scheme shown in Fig. 5 electron acceptors in chloroplast reactions may be divided into those which react in position B and those which react in position A. Only the first group, exemplified by TPN, would give a coupled photophosphorylation. 2,3′,6-trichlorophenol indophenol is an example of the second group. It is possible, however, that certain compounds although reacting preferentially in one position may also be able to react in the other.

Since indophenol dyes are reduced by illuminated chloroplasts much more rapidly than ferricyanide (Witt *et al.*, 1956; Krogmann & Jagendorf, 1959), a catalytic amount of the dye can convert noncyclic photophosphorylation with ferricyanide into a photo-oxidation of water reaction (Table 2). In the absence of the dye ferricyanide acts as a terminal electron acceptor B in noncyclic photophosphorylation; oxygen evolution is accompanied by ATP formation (cf. Fig. 5 and Table 2, Treatments 1 and 3). When the dye is present, we envisage it as

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acting in position A in Fig. 4. It is repeatedly reduced during the photooxidation of water and then reoxidized by ferricyanide in the dark. The overall result is also oxygen evolution but without ATP formation (cf. Fig. 4 and Table 2, Treatments 2 and 4). The addition of the dye cannot have a similar effect in the TPN system because here the dark reoxidation of the reduced dye by TPN is precluded by the difference in the respective normal redox potentials (0·217 V for 2,6-dichlorophenol indophenol and −0·324 V for TPN). Photoreduced indophenol dyes cannot, therefore, be reoxidized by TPN in the dark and thereby convert noncyclic photophosphorylation with TPN into a photooxidation of water reaction (Table 3).

TABLE 3—EFFECT OF INDOPHENOL DYES ON NONCYCLIC PHOTOPHOSPHORYLATION BY CHLOROPLASTS IN THE PRESENCE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE (TPN)

No.	Treatment	Effective electron acceptor	Oxygen produced (μatom)	Adenosine triphosphate formed (μmole)
1	TPN	TPN	3·3	3·2
2	Dye A plus TPN	TPN	3·1	4·2
3	TPN	TPN	3·0	3·3
4	Dye B plus TPN	TPN	3·1	3·6

Experimental conditions were the same as for Table 2, except that the ferricyanide was omitted. 4 μmole TPN and 0·1 ml of a purified preparation of triphosphopyridine nucleotide reductase from spinach were added in each treatment.

We have not identified the "pigment", shown in Figs. 4 and 5, that is involved in the photooxidation of water. Recent experiments (Arnon *et al.*, 1961a) on the effectiveness of monochromatic light on the photochemical reactions of chloroplasts suggest that this pigment is more likely to be chlorophyll or an accessory pigment that is found only in higher plants and algae, rather than chorophyll-a (or the closely related bacterio-chlorophyll) found in all photosynthetic cells (Losada *et al.*, 1961). In the red region of the spectrum the photooxidation of water was most effective around 644 which corresponds to the maximum absorption peak (French, 1960) in the red for chlorophyll-b (Figs. 6 and 7). The effectiveness of monochromatic light in the red region on photoreduction of TPN (equation (5)) and cyclic photophosphorylation (equation (1)) was distinctly different from that on oxygen evolution (equation (4) and Figs. 6 and 7), thus supporting the view that these are different light reactions. The experiments with monochromatic light favor the conclusion that the photochemical reaction which produces oxygen is different from the other photochemical reactions which produce ATP and reduced pyridine nucleotide.

The current concept of noncyclic photophosphorylation in green plants presented here is a further elaboration, in the light of new experimental findings, of the one presented earlier (Arnon, 1959). It is now possible to envisage in more detail the electron transfer reactions from water (OH^-) that result in oxygen evolution.

We had first suggested that this may be a dark reaction between water (OH^-) and a cytochrome component peculiar to green plants (Arnon, 1959). Such a reaction, although it appeared thermodynamically possible (Arnon, 1959), could not be

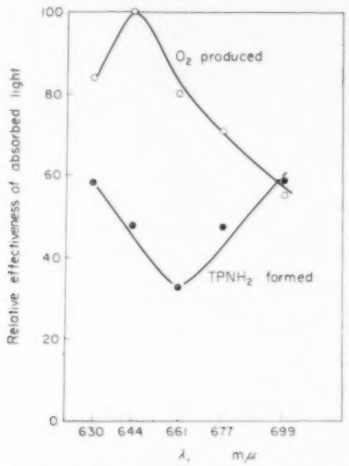

FIG. 6. Effectiveness of monochromatic light, in the red region of the spectrum, on oxygen evolution and TPN reduction occurring as separate chloroplast reactions by isolated chloroplasts.

Oxygen was measured manometrically when the photochemical reaction was limited to the photo-oxidation of OH^- (equation (4)) in a reaction mixture which included, in a final volume of 3 ml, chloroplast fragments (C_{ls}) containing 1 mg of chlorophyll, and the following in μmole: Tris acetate buffer, pH 8, 40; potassium ferricyanide, 15; and 2,6-dichlorophenol (DCPIP), 0·2. The reaction was run for 15 min at 15°C. Gas phase, nitrogen. TPN reduction was carried out in a parallel experiment in which the photooxidation of water was blocked by the omission of chloride and the addition of 2×10^{-5} M CMU. The electron donor system (see A^- in equation (5)) for TPN reduction (measured spectrophotometrically at 340 mμ) consisted of 20 μmoles ascorbate and 0·05 μmole of 2,3,6-trichlorophenol indophenol. 100 on the ordinate scale is equivalent to 0·15 μatom oxygen evolved per μmole quanta of light absorbed. 58 on the ordinate scale is equivalent to 0·09 μmole $TPNH_2$ formed per μmole quanta of light absorbed.

described in terms of the properties of the presently known cytochrome constituents of green cells (Hill & Bendall, 1960). We next suggested (Arnon, 1961a, b) that the interaction between OH^- and cytochromes may require an additional input of energy and now it appears that this energy is supplied by an auxiliary light reaction. This auxiliary light reaction is found in green plants but not in photosynthetic bacteria, and explains why photosynthesis is accompanied by an evolution of oxygen only in green plants.

ROLE OF OXYGEN IN PHOTOSYNTHETIC PHOSPHORYLATION

When photosynthetic phosphorylation was first discovered it proceeded at a sustained rate only in the presence of oxygen (Arnon *et al.*, 1954). However, since

there was no net consumption (as measured by manometric pressure change) of molecular oxygen, we concluded then that oxygen acted as catalyst in photosynthetic phosphorylation, not as a substrate as in oxidative phosphorylation. A decisive

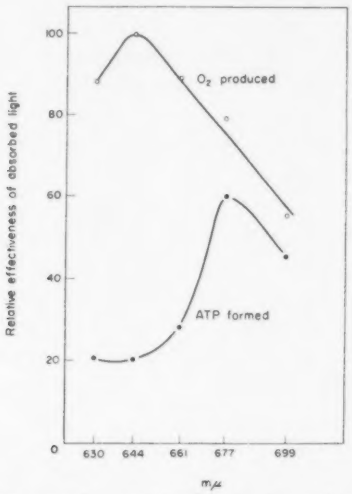

FIG. 7. Effectiveness of monochromatic light, in the red region of the spectrum, on oxygen evolution and cyclic photophosphorylation, occurring as separate chloroplast reactions by isolated chloroplasts.

Oxygen evolution was measured as described in Fig. 6. ATP formation by cyclic photophosphorylation (equation (1)) was measured in a parallel experiment in a reaction mixture which included, in a final volume of 3 ml, chloroplast fragments (C_{ls}) containing 1 mg chlorophyll and the following in μmole: Tris/HCl buffer, pH 8·3, 80; $MgSO_4$, 5; ADP, 10; $K_2HP^{32}O_4$, 10; and vitamin K_3 (menadione), 0·3. 100 on the ordinate scale is equivalent to 0·16 μatom oxygen evolved per μmole quanta of light absorbed. 60 on the ordinate scale is equivalent to 0·10 μmole ATP formed per μmole quanta of light absorbed.

difference between photosynthetic and oxidative phosphorylation, then and now, was the inability of chloroplasts to form ATP in the dark by oxidizing substrates of oxidative phosphorylation with molecular oxygen (Arnon, 1955; Arnon *et al.*, 1956).

The catalytic action of oxygen may now be explained by the proposed mechanism for noncyclic photophosphorylation (Fig. 5). Oxygen, when present in the reaction mixture, can replace TPN as the electron acceptor B (this electron transfer is greatly accelerated by the addition of "Micro-catalytic" amounts of vitamin K or FMN (Wessels, 1958; Jagendorf & Avron, 1959; Nakamato *et al.*, 1959; Arnon, 1961b; Arnon *et al.*, 1954)). The substitution of oxygen is analogous to the substitution of ferricyanide for TPN but is accompanied by an even greater loss of energy. The energy loss here is equivalent to a redox potential of 1139 mV (difference between $E^0 = 0{\cdot}815$ V for the water/oxygen system and $E^0 = -0{\cdot}324$ V for the TPN/$TPNH_2$ system). ATP is formed in each case but the "reductant" produced is now water instead of $TPNH_2$.

If, at the same time that oxygen is used as the electron acceptor B, water (OH^-) is used as the electron donor (bottom of Fig. 5), the resultant oxygen evolution at one end would balance the oxygen consumption at the other. An oxygen exchange reaction would result, without any net consumption of oxygen that could be measured manometrically; oxygen would act as a catalyst.

The catalytic effect of oxygen would also explain why oxygen accelerates photosynthetic phosphorylation when FMN and vitamin K are not added at all (Arnon *et al.*, 1954) or are added in concentrations that are suboptimal for cyclic photophosphorylation (Wessels, 1958; Jagendorf & Arnon, 1959; Nakamato *et al.*, 1959; Arnon, 1961b). Moreover, because of its high affinity for electrons donated by reduced FMN or reduced vitamin K, oxygen, even when introduced to an anaerobic cyclic photophosphorylation system that is catalyzed by an adequate amount of FMN or vitamin K, converts these anaerobic systems into an oxygen catalyzed type (Arnon, 1961b). Photophosphorylation now also becomes dependent on the flow of electrons from water (OH^-) and its concomitant oxygen evolution.

Experimental support for this interpretation was found by comparing aerobic and anaerobic photophosphorylation systems under conditions when they responded optimally to the addition of FMN or vitamin K. A marked difference was observed, depending on the presence or absence of oxygen, in the effect of two inhibitors, CMU (p-chlorophenyldimethylurea) and *o*-phenanthroline. The results are shown in Table 4.

In agreement with the findings of Wessels (1958), Jagendorf & Avron (1959) and Nakamoto *et al.* (1959) *o*-phenanthroline and CMU, in the presence of air, inhibited photophosphorylation in the FMN and vitamin K systems. But relatively little inhibition by these two inhibitors was observed in an atmosphere of nitrogen. In other experiments, not reported here, we found phenazine methosulfate to differ from FMN and vitamin K in that its pathway was resistant to inhibition by *o*-phenanthroline and CMU, either in air or in nitrogen.

o-phenanthroline and CMU are powerful inhibitors of oxygen evolution by illuminated chloroplasts. It seems likely therefore, that these inhibitors acted by impeding the photoproduction of oxygen, which under aerobic conditions becomes a component step in photophosphorylations catalyzed by FMN or vitamin K. This conclusion is also supported by the observed effect of chloride on photophosphorylation with vitamin K and FMN in air and in nitrogen (Table 5). Chloride is required for the photoproduction of oxygen by isolated chloroplasts (Warburg, 1949). The omission of chloride had scarcely an effect on photophosphorylation in nitrogen, but it inhibited severely photophosphorylation in air, when, in accordance with our scheme, photoproduction of oxygen would become necessary to maintain the electron flow.

These results are interpreted to mean that oxygen, when present in a system catalyzed by either FMN or vitamin K, is able to compete effectively and trap the electrons of cyclic photophosphorylations. Once the electrons are accepted by oxygen and form water, an oxygen-dependent, "pseudocyclic" mechanism (Arnon *et al.*, 1961a) takes over because the flow of electrons can be maintained only by a

release of electrons through photooxidation of OH^- (Fig. 5). By contrast, phenazine methosulfate catalyzes the cyclic flow of electrons so effectively that it is able to prevent their capture by oxygen and hence cyclic photophosphorylation catalyzed by phenazine methosulfate (Fig. 2) remains "anaerobic" even when molecular oxygen is present.

TABLE 4—EFFECT OF *o*-PHENANTHROLINE (O-P) AND DICHLOROPHENYL-DIMETHYL-UREA (CMU) ON CYCLIC PHOTOPHOSPHORYLATION IN NITROGEN OR AIR

Treatment	Percent inhibition	
	CMU	O–P
Nitrogen FMN	25	20
Nitrogen, vitamin K_3	19	27
Air, FMN	97	77
Air, vitamin K_3	85	64

In the nitrogen series the illumination was 2000 Lux for 30 min and the reaction mixture included, in a final volume of 3 ml, chloroplast fragments (C_{1a}) containing 1·5 mg chlorophyll and 0·3 μmole of FMN or vitamin K_3. In the air series the illumination was 50,000 Lux for 5 min and the reaction mixture included chloroplast fragments (C_{1a}) containing 1 mg chlorophyll and 0·0003 μmole of FMN or vitamin K_3. The final inhibitor concentrations were 3×10^{-5} M for *o*-phenanthroline and 2×10^{-6} M for CMU. Other common components of the reaction mixture were, in μmoles: Tris buffer, pH 8·3; $K_2HP^{32}O_4$, 15; $MgSO_4$, 5; and ADP, 15.

TABLE 5—EFFECT OF CHLORIDE ON CYCLIC PHOTOPHOSPHORYLATION IN NITROGEN OR AIR

Treatment	μmole ATP formed	
	minus chloride	plus chloride
Nitrogen, FMN	5·1	5·7
Nitrogen, vitamin K_3	9·7	9·9
Air, FMN	0·5	6·1
Air, vitamin K_3	0·4	5·5

Experimental conditions as in Table 4, except that chloroplasts were prepared in 0·5 M sucrose and chloride-free reagents were used. 0·2 mg chlorophyll was used in the air series, and 2·5 mg chlorophyll per vessel was used in the nitrogen series. The reaction was run for 30 min.

Molecular oxygen can also serve as the terminal electron acceptor B (Fig. 3) in an experimentally contrived system in which the use of water as the electron donor is blocked by inhibitors and an external electron donor is supplied instead. Krogmann & Vennesland (1959) have described a system of this kind in which ATP formation was accompanied by the simultaneous consumption of O_2 and of an external electron donor. An apparent "oxidative photosynthetic phosphorylation"

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resulted that resembled oxidative phosphorylation by mitochondria, especially when $DPNH_2$ was used as the electron donor. However, the analogy between such "oxidative photosynthetic phosphorylation" and oxidative phosphorylation by mitochondria appears to be fortuitous. The role of $DPNH_2$ in this chloroplast system was not that of a physiological electron donor but that of a nonspecific reducing agent for the dye, one of several reducing agents that were effective. That the consumption of oxygen was artificially induced, and was not a physiological component of photophosphorylation, is made clear by the observations of Krogmann & Vennesland (1959) that "there was ample energy released by the dark oxidation of the DPNH to form the high energy phosphate bonds. Nevertheless, the reaction gave no phosphorylation unless the system was illuminated, even though the light caused no increase in the rate of oxygen consumption."

To recapitulate, cyclic photophosphorylation is an anaerobic process (Arnon *et al.*, 1961a) that proceeds without the consumption of molecular oxygen. In photosynthetic bacteria the question of oxygen consumption does not arise since their photosynthesis is strictly anaerobic. In isolated chloroplasts anaerobic cyclic photophosphorylation occurs in the presence of phenazine methosulfate, or of suitable concentrations of vitamin K or FMN. Oxygen when present jointly with FMN or vitamin K (but not with phenazine methosulfate) competes effectively for electrons in the cycle (Fig. 1) and thus becomes an electron trap in photophosphorylation by isolated chloroplasts. The participation of oxygen in photosynthetic phosphorylation by isolated chloroplasts is an energy-wasteful step and there is no evidence that it occurs under physiological conditions. Isolated chloroplasts do not respire—a conclusion which has recently received strong support from the work of James & Das (1957).

As already discussed, in noncyclic photophosphorylation by chloroplasts the formation of ATP is coupled with the evolution of oxygen and the reduction of TPN (replaceable by ferricyanide). (This is the reverse of oxidative phosphorylation by mitochondria where ATP formation is coupled with the consumption of oxygen and oxidation of reduced pyridine nucleotide.) The electron transport in chloroplasts, as measured by the rates of ferricyanide reduction and of oxygen evolution, was found to be markedly increased, when it is coupled with ATP formation (Arnon *et al.*, 1958; Avron, 1958). Davenport (1960) has significantly extended the evidence for this by finding an increase in the rate of photoreduction of TPN by isolated chloroplasts when this reaction was coupled with ATP formation. However, Warburg *et al.* (1959) using β-naphthoquinonesulfonic acid or ferricyanide as oxidants, found no influence of the phosphorylating system on the rate of photoproduction of oxygen by isolated chloroplasts, and have attributed the contrary results of other investigators to fluctuations in pH—a possibility which was ruled out by recent experiments (Arnon *et al.*, 1961a).

NONCYCLIC PHOTOPHOSPHORYLATION IN BACTERIAL PREPARATIONS

Until recently, cyclic photophosphorylation was the only experimentally demonstrated type of photosynthetic phosphorylation in photosynthetic bacteria.

Since photosynthetic bacteria cannot use water (OH^-) as an electron donor, they would not be expected to have a noncyclic photophosphorylation reaction of the chloroplast type (equation (2)) in which ATP formation and pyridine nucleotide reduction are coupled with oxygen evolution, but there was no theoretical reason why photosynthetic bacteria could not carry out the primary noncyclic photophosphorylation (Fig. 3) in which electron donors other than water (OH^-) are used. Photoreduction of DPN by cell-free preparation of *R. rubrum* (Frenkel, 1958; Vernon & Ash, 1959) and *Chromatium* (Ogata *et al.*, 1959) has been observed, but attempts to find in chromatophores of *R. rubrum* a coupled ATP formation using succinate or ascorbate as electron donors led to negative results (Frenkel, 1958; Vernon & Ash, 1960).

Evidence for noncyclic photophosphorylation in chromatophores of *R. rubrum* has recently been obtained by Nozaki *et al.* (1961), under conditions when cyclic photophosphorylation was suppressed and ATP formation was obligatorily coupled with a light-dependent electron flow from an external electron donor to DPN as the terminal electron acceptor. Without suppressing cyclic photophosphorylation, the ATP formed by this mechanism could not be distinguished from the ATP formed by the noncyclic mechanism.

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TABLE 6—INHIBITORS OF CYCLIC PHOTOPHOSPHORYLATION IN CHROMATOPHORES OF *R. rubrum*

Inhibitor added	μmole ATP formed/mg chl/hr
None	300*
Antimycin A	5·0
HOQNO, 10^{-5} M	15·3

* Reaction became limited by exhaustion of ADP.

The reaction mixture contained in a final volume of 3 ml: chromatophores, containing 0·1 mg bacterio-chlorophyll; hexokinase, 1 mg; and the following in μmoles: tris buffer, pH 7·9, 100; $MgCl_2$, 5; ADP, 10; $K_2HP^{32}O_4$, 10; D-glucose, 80. The following inhibitors were added, where indicated: antimycin A, 10 μg; 2-heptyl-4 hydroxyquinoline-N-oxide (HOQNO), 0·03 μmole. The reactions were run at 20° for 30 min. Gas phase, argon; illumination, 10,000 Lux.

Table 6 shows that, as was already found by other investigators (Geller, 1957; Geller & Lipman, 1960; Smith & Baltscheffsky, 1959; Baltscheffsky & Baltscheffsky, 1960), antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) (a gift of Dr. D. L. Hackett) inhibited cyclic photophosphorylation by *R. rubrum* chromatophores. These two substances were also found to inhibit photoreduction of DPN when succinate was the electron donor (Fig. 8). Succinate could not, therefore, be used as the electron donor for noncyclic photophosphorylation in the presence of any one of these inhibitors. However, HOQNO and antimycin A gave practically no inhibition when ascorbate, together with catalytic amounts of

2,6-dichlorophenol indophenol (DPIP), served as the electron donor for DPN reduction (Fig. 8). It thus became possible to measure ATP formation that is coupled with the electron flow from ascorbate to DPN, under conditions when cyclic photophosphorylation was blocked.

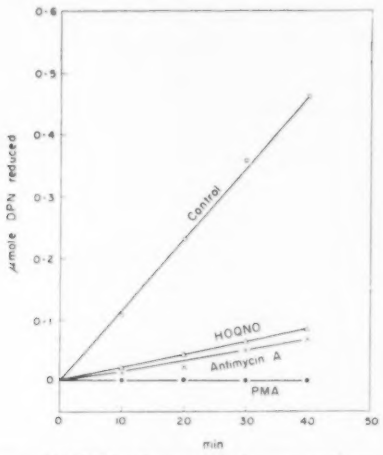

FIG. 8. Photoreduction of DPN by bacterial chromatophores with succinate. The reaction mixture contained, in a total volume of 3 ml, 0·03 mg bacteriochlorophyll as washed *R. rubrum* chromatophores and, in μmoles: Tris buffer (pH 7·9), 100; $MgCl_2$, 5; DPN, 2; and succinate, 20. The following inhibitors were added as indicated; antimycin A, 10 μg; 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), 0·03 μmole; or phenyl mercuric acetate (PMA), 0·3 μmole. The reaction was carried out in Thunberg type spectrophotometer cells at 20°C. Gas phase, argon; illumination, 10,000 Lux.

As shown in Table 7, a noncyclic photophosphorylation system was functioning with chromatophores from *R. rubrum*. The formation of ATP required the simultaneous presence of an electron donor (ascorbate + DPIP) and of an electron acceptor (DPN). The stoichiometry of this reaction appears to be the same as in noncyclic photophosphorylation by isolated chloroplasts; one mole of ATP is formed for each mole of pyridine nucleotide reduced (Fig. 10). Operationally, this stoichiometry was obtained only when the reduced DPN was trapped by an added lactic dehydrogenase system. Without this addition, the reduced DPN was reoxidized in the reaction mixture, resulting in low recoveries of $DPNH_2$ and high $P/DPNH_2$ ratios.

A diagrammatic representation of noncyclic photophosphorylation in bacteria is given in Fig. 11. It seems likely that electron transport from succinate to DPN shares with the electron transport system of cyclic photophosphorylation the same step, sensitive to inhibition by antimycin A and HOQNO, that involves electron transfer between a cytochrome of the b type and a cytochrome of the c type (Smith & Baltscheffsky, 1959; Baltscheffsky & Baltscheffsky, 1960). A possible participation

of coenzyme Q in the sector of the electron transport chain in bacterial photophosphorylations is discussed elsewhere (Baltscheffsky & Baltscheffsky, 1960).

TABLE 7—NONCYCLIC PHOTOPHOSPHORYLATION IN *R. rubrum*

Additions	μmole ATP formed/mg chl/hr
None	5·0
Ascorbate, DPIP	5·4
DPN	7·6
DPN, ascorbate, DPIP	44·8

All vessels contained antimycin A, 10 μg. The following additions were made where indicated: ascorbate, 20 μmoles; 2,6-dichlorophenol indophenol (DPIP), 0·2 μmole; DPN, 2 μmoles. Other components of the reaction mixture and conditions were the same as in the control treatment of Table 6.

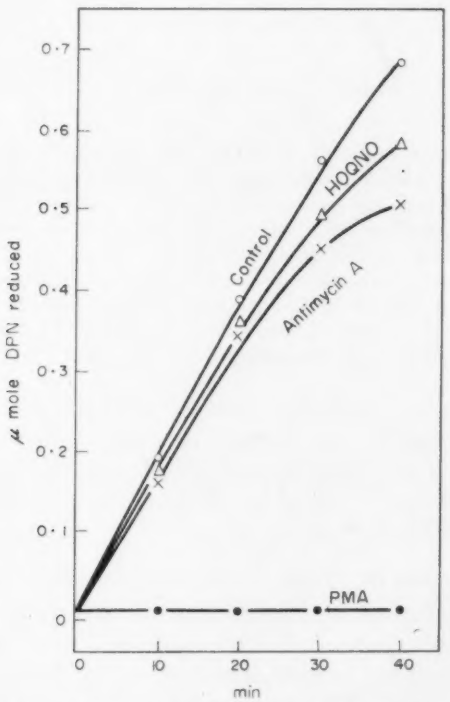

FIG. 9. Photoreduction of DPN by bacterial chromatophores with ascorbate. The reaction mixture and experimental conditions were the same as described for Fig. 8 except that 20 μmoles of ascorbate and 0·2 μmole of 2,6-dichlorophenol indophenol were added instead of succinate.

Additional support for noncyclic electron flow in photosynthetic bacteria has come from recent experiments of Losada *et al.* (1961) and Arnon *et al.* (1960, 1961a) on photoproduction of H_2 and photofixation of N_2 by *Chromatium* at the

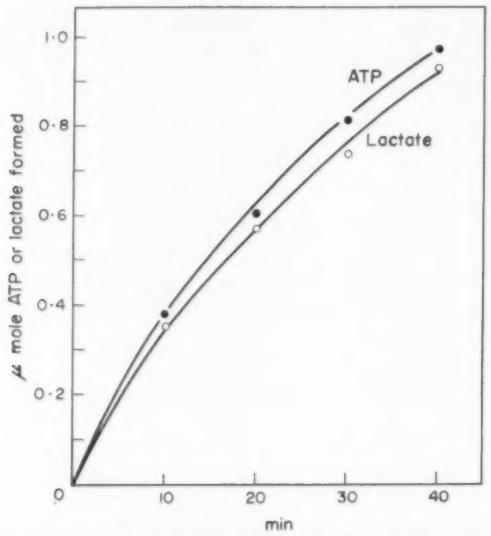

FIG. 10. Stoichiometry of ATP formation and DPN reduction to (as measured by lactate formation) in noncyclic bacterial photophosphorylation. The reaction mixture contained 0·04 mg bacteriochlorophyll as washed chromatophores, and in μmoles: Tris buffer (pH 7·9), 100; $MgCl_2$, 5; ADP, 5; $K_2HP^{32}O_4$, 5; ascorbate, 20; 2,6-dichlorophenol indophenol, 0·2; DPN, 0·3; pyruvate, 10; and 25 μg of crystalline lactic dehydrogenase (muscle) and 10 μg of antimycin A. The reaction was carried out in Thunberg tubes, at 20°C. Gas phase, argon; illumination, 10,000 Lux. The formation of lactate was measured by a modified method of S. B. Barker and W. H. Summerson (*J. Biol. Chem.* (1941) **138**, 535).

expense of thiosulfate as the electron donor. The results are consistent with the view that electrons expelled from chlorophyll, following the primary photochemical act, are responsible for the reduction of H+ to H_2 and for the reduction of N_2. The electrons lost by chlorophyll would be replaced by those donated by thiosulfate, via the bacterial cytochrome system.

LIGHT REACTIONS IN PLANT AND BACTERIAL PHOTOSYNTHESIS

Since noncyclic photophosphorylation in bacteria (Fig. 11) is analogous to noncyclic photophosphorylation without oxygen evolution in chloroplasts (Fig. 3), photosynthesis in plants and bacteria is now seen as having in common two photochemical processes: cyclic and noncyclic photophosphorylation. In both plants and bacteria light energy may serve a dual purpose. It supplies ATP by cyclic and by noncyclic photophosphorylation, and it brings about a reduction of pyridine nucleotide and oxidation of an exogenous electron donor that is coupled with the

formation of ATP. In organisms which contain, or can adaptively form, hydrogenase or nitrogenase (photosynthetic bacteria and algae), the noncyclic electron flow can also be observed as a photoproduction of molecular hydrogen or a photofixation of nitrogen gas.

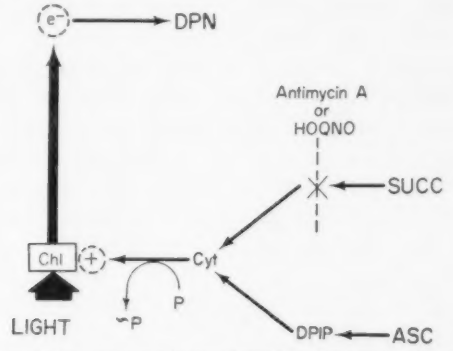

FIG. 11. Non-cyclic photophosphorylation in *R. rubrum*.

The noncyclic eiectron flow mechanism in photosynthetic bacteria operates with such electron donors as reduced sulfur compounds or organic acids. Water (OH^-) became an electron donor only with the emergence of plant photosynthesis. It now appears that this event was made possible because plants have evolved an auxiliary light-dependent reaction in which the electrons from water (OH^-) are first raised to a potential similar to that of the electron donors in bacterial photosynthesis, and are then used in a second photochemical reaction—the primary photophosphorylation reaction common to plants and bacteria—in which pyridine nucleotide reduction is coupled with the formation of ATP (Fig. 3).

The involvement of two light reactions with the participation of water in the photosynthetic cytochrome system of both green plants and photosynthetic bacteria has been postulated by Hill & Bendall (1960). However, our findings support the conclusion that photooxidation of water is peculiar to green plants and absent in photosynthetic bacteria. Oxygen evolution is peculiar to green plants because it is the product of the photooxidation of water (OH^-), that is found only in green plants. Water as an electron donor is, therefore, not involved in the two main events of noncyclic photophosphorylation in plants and bacteria, the photoreduction of pyridine nucleotide and the coupled formation of ATP.

In summary, the differences between bacterial and plant photosynthesis seem to center on the electron donors that are consumed in the reduction of pyridine nucleotide. With some bacterial electron donors, as for example hydrogen gas, the reduction of pyridine nucleotide by photosynthetic bacteria requires no input of light energy. With other electron donors, such as thiosulfate or succinate, an input of light energy, in a noncyclic electron flow mechanism, becomes necessary for the reduction of pyridine nucleotide. Thus in bacterial photosynthesis, the reduction

of pyridine nucleotide may occur with or without an input of light energy depending on the particular electron donor used. By contrast, in photosynthesis of green plants, the reduction of pyridine nucleotide invariably requires an input of light energy because the physiological electron donor is water (OH^-).

ENERGY CONVERSION AS A UNIFYING CONCEPT IN PHOTOSYNTHESIS

The concept of photosynthesis to which we were led in the 6 years since the process was first completely localized in isolated chloroplasts differs from the conventional view that photosynthesis is mainly a process of CO_2 assimilation. Photosynthesis appears to be first and foremost a process for converting sunlight into chemical energy and this conversion is more directly associated with phosphorus than with carbon assimilation. In the light of present knowledge, photosynthesis may be defined as the synthesis of cellular substances at the expense of chemical energy formed by photochemical reactions. This definition includes, but is not limited to, CO_2 assimilation.

In both bacterial and plant photosynthesis the photochemical reactions proper are limited to the formation of ATP and reduced pyridine nucleotide by cyclic and noncyclic photophosphorylation. This transformation of light into the common currency of cellular energy is fundamentally independent of CO_2 assimilation. This is no particular reason why ATP or a photochemically generated reductant could not be used for driving endergonic cellular processes other than CO_2 assimilation.

The photo-assimilation of acetate by *Chromatium* (Losada *et al.*, 1960) and by *Chlorella* and other green algae (Pringsheim & Wiessner, 1960), under conditions when CO_2 assimilation was excluded, are cases of photosynthesis without either oxygen evolution or CO_2 reduction. So is the light-dependent conversion of glucose into starch (Maclachlan & Porter, 1959). In the conventional view of photosynthesis this state of affairs would be a contradiction of terms, but according to our present concept these examples represent photosynthesis because they involve biochemical syntheses that are being driven by light energy after light has been converted by the cell into ATP.

In this view of photosynthesis, CO_2 assimilation, although quantitatively the dominant form of photosynthesis on our planet, is fundamentally only a special case of the use and storage of light energy. CO_2 assimilation proper, in both green plants and photosynthetic bacteria, consists of exclusively dark reactions that are not peculiar to photosynthesis, a conclusion that was also reached by investigators of the carbon path in photosynthesis. Thus Calvin (1959) wrote recently, "The reduction of carbon dioxide, we now have every reason to suppose, occurs in a series of reactions which can take place entirely in the dark. In fact, all the enzyme systems that we now know participate in the conversion of CO_2 to carbohydrates have been found in a wide variety of organisms, many of which are not photosynthetic."

The familiar accumulation of carbon compounds as carbohydrates during photosynthesis in green plants constitutes storage of trapped light energy and is

fundamentally extraneous to the energy conversion process itself. The first chemically identified products of photosynthesis in green plants are now known to be ATP and $TPNH_2$. They are present, however, in the cell only in catalytic amounts and cannot be accumulated in any appreciable amount for future use; they are promptly consumed in the synthesis of carbohydrates, fats, proteins and other cellular constituents.

The proposal that ATP formation is a fundamental event in photosynthesis has been made earlier, notably by Emerson *et al.* (1944) but, as was recently pointed out by Umbreit (1960), "the early experiments were not adequate to demonstrate it". Without sufficient experimental evidence, the early theoretical proposals of Umbreit and his associates could not be adequately defended against the theoretical objections leveled against them (as, for example, by Rabinowitch (1945) particularly since several years later the first experiments with P^{32} to test the occurrence of light-induced phosphorylation in cell-free systems led to negative results. Aronoff and Calvin, who made these experiments with spinach grana, reported that "there is no direct connection between light and the gross formation of organic phosphorus compounds" (Aronoff & Calvin, 1948)).

Photosynthetic phosphorylation has now been found in every type of photosynthetic organism tested: higher green plants, algae and photosynthetic bacteria. The conclusion seems, therefore, justified that photosynthetic phosphorylation represents a fundamental process, common to all photosynthetic organisms, by which light energy is converted into chemical energy.

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MECHANISM OF UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY NITROPHENOLS

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INTRODUCTION

ALL oxidative phosphorylation reactions can be described by the equation

$$AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP$$

The energy required for the phosphorylation of ADP by P_i

$$ADP + P_i \rightleftharpoons ATP - 8000 \text{ cal } (\Delta G'_o)$$

is supplied by hydrogen or electron transfer from the $AH_2 \rightleftharpoons A$ system at lower oxidation-reduction potential to $BH_2 \rightleftharpoons B$ at higher potential.

We distinguish between two types of oxidative phosphorylation:

(a) *substrate-linked*, where AH_2 is one of the substrates phosphoglyceraldehyde, pyruvate or α-ketoglutarate;

(b) *respiratory-chain*, where AH_2 and B are both members of the respiratory chain.

Studies of the enzyme systems bringing about substrate-linked phosphorylation, which are more amenable to direct experimentation than those catalysing respiratory-chain phosphorylation, have given evidence that a high-energy intermediate is formed before the intervention of P_i or ADP, as described in equations (1) and (2) (Slater (1958) for fuller discussion).

$$AH_2 + B + C \rightleftharpoons A \sim C + BH_2 \quad (1)$$

$$A \sim C + ADP + P_i \rightleftharpoons A + C + ATP \quad (2)$$

$$\textit{Sum:}\ \overline{AH_2 + B + ADP + P_i \overset{(C)}{\rightleftharpoons} A + BH_2 + ATP} \quad (3)$$

In every case C is an $-SH$ compound.

The detailed mechanisms of reactions (1) and (2) which have been proposed differ according to the substrate, thus

Reaction (1)

phosphoglyceraldehyde
$$\begin{cases} B + C \rightleftharpoons BC \\ BC + AH_2 \rightleftharpoons A \sim C + BH_2 \end{cases}$$

pyruvate, α-ketoglutarate
$$\begin{cases} AH_2 + B \rightleftharpoons A \sim BH_2 \\ A \sim BH_2 + C \rightleftharpoons A \sim C + BH_2 \end{cases}$$

Abbreviations: ADP, ATP, adenosine diphosphate and triphosphate; GDP, guanosine diphosphate; P_i, inorganic phosphate; As_i, inorganic arsenate; DPN^+, DPNH, oxidized and reduced diphosphopyridine nucleotide; fp, fpH_2, oxidized and reduced flavoprotein; DNP, 2,4-dinitrophenol.

Reaction (2)

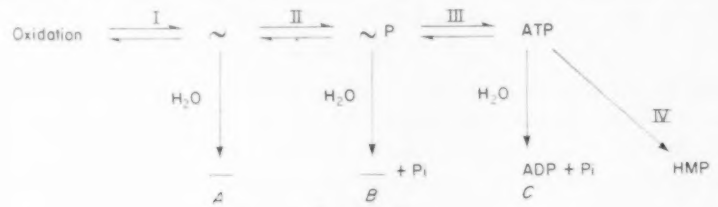
phosphoglyceraldehyde, pyruvate $\begin{cases} A \sim C + P_i \rightleftharpoons A \sim P + C \\ A \sim P + ADP \rightleftharpoons A + ATP \end{cases}$

α-ketoglutarate $\begin{cases} A \sim C + P_i \rightleftharpoons C \sim P + A \\ C \sim P + ADP \overset{(GDP)}{\rightleftharpoons} C + ATP \end{cases}$

In every case, P_i reacts before ADP. There is good evidence that this is also the case with respiratory-chain phosphorylation (reviewed in Slater, 1958).

In 1953 (Slater), it was suggested that respiratory-chain phosphorylation could also be described by the same two equations (1) and (2). Many of the formulations of oxidative phosphorylation in use at present follow this suggestion, with or without modification (see Slater, 1958 for fuller discussion).

Reactions (1) and (2) can be described by the top line of Scheme I



SCHEME I.

Since the high-energy compound (A ~ C) is a bound or inhibited form of one of the catalysts of the oxidation reaction, it is necessary to discharge the ~ to remove the inhibition of the oxidation reaction (Chance & Williams, 1956). This can be done by adding sufficient P_i and ADP to drive reactions (1) and (2) towards ATP formation. A constant concentration of ADP can be assured by adding glucose and hexokinase which brings the system down to a lower energy level. The inhibition of the oxidation reaction will also be relieved, and the esterification of P_i prevented, by bringing about the hydrolysis of any one of the three high-energy compounds, as shown at points *A*, *B* and *C* in Scheme I. If hydrolysis is introduced at any one of these points, the reversible oxidative phosphorylation reaction (3) will be replaced by the non-phosphorylating irreversible reaction (4),

$$AH_2 + B \rightarrow A + BH_2 \qquad (4)$$

Such a system is said to be "uncoupled".

Hydrolysis at point *C* can be brought about by the addition of ATPase. In this case, both P_i and ADP are necessary as catalysts for reaction (4), but there is no net ATP formation. This is probably important *in vivo*, since an ATP-utilizing reaction is often in effect an ATPase. *In vitro*, uncoupling at point *C* can usually be prevented by adding sufficient hexokinase to compete with the ATPase. There is evidence, however, that under some conditions the ATP can be formed in a "compartment" in the mitochondria where it is more accessible to a mitochondrial ATP-utilizing reaction than to hexokinase which is outside the mitochondria (see, for example, Slater & Hülsmann, 1961).

Hydrolysis at point *B* can be effected by addition or activation of a phosphatase, e.g. the addition of acyl phosphatase (Harary, 1957) which catalyses the hydrolysis of diphosphoglyceric acid, the ~ P intermediate in the substrate-linked phosphorylation with phosphoglyceraldehyde. In this case, P_i but not ADP is necessary for the catalysis of reaction (4).

Hydrolysis at point *A* can be effected, in the case of substrate-linked phosphorylation, by the addition of arsenate (As_i) which can replace P_i in the phosphorolysis of A ~ C, thus

$$A \sim C + As_i \rightleftharpoons A \sim As + C$$

The arsenate compound is very unstable and reacts immediately with water, thus

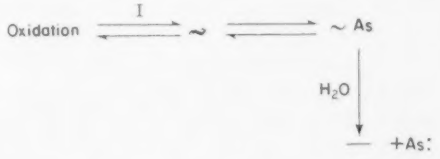
$$A \sim As + H_2O \rightarrow A + As_i$$

The sum of these two reactions is the arsenate-catalysed hydrolysis of A ~ C

$$A \sim C + H_2O \xrightarrow{(As_i)} A + C$$

Arsenate can replace the need for both P_i and ADP in reaction (4). The same result can be obtained by adding an A ~ Case (e.g. succinyl deacylase (Kaufman, 1951).

Uncoupling by arsenate can be schematically represented in terms of Scheme I by



If reactions (iii) and (ii) in Scheme I are reversible, the introduction of a hydrolytic reaction at either *B* or *A* will endow the system with the ability to hydrolyse added ATP, thus

$$\begin{array}{l} ATP + A \rightleftharpoons A \sim P + ADP \\ A \sim P + H_2O \longrightarrow A + P_i \\ \hline ATP + H_2O \xrightarrow{(A)} ADP + P_i \end{array}$$

or

$$\begin{array}{l} ATP + A + C \rightleftharpoons A \sim C + ADP + P_i \\ A \sim C + H_2O \longrightarrow A + C \\ \hline ATP + H_2O \xrightarrow{(A,C)} ADP + P_i \end{array}$$

The first reaction sequence has been demonstrated when A = 3-phosphoglyceric acid, in the presence of phosphoglyceric kinase and acyl phosphatase (Harary, 1957). The second sequence has been demonstrated with A = succinate, C = coenzyme A in the presence of "P" enzyme and succinyl-CoA deacylase (Kaufman, 1951). It has also been demonstrated when arsenate is added to

hydrolyse A ~ C, both with glycolytic enzymes and with mitochondria (Wadkins, 1960). Arsenate is a general uncoupling agent, being active against both substrate-linked and respiratory-chain phosphorylation.

The classical uncoupler of respiratory-chain phosphorylation is 2,4-dinitrophenol (Loomis & Lipmann, 1948), which has no effect on substrate-linked phosphorylation. Although this was not directly demonstrated until 1948, it was known more than 10 years previously that nitrophenols stimulated the respiration of intact cells, while at the same time they inhibited various energy-requiring functions of the cells, and Lardy & Elvehjem (1945) had already suggested that these compounds act either by allowing oxidation to proceed without phosphorylation, or by catalysing the hydrolysis of an intermediate phosphate compound. All explanations of the action of dinitrophenol which have been proposed since 1945 can be considered modifications or extensions of one of these two fundamental hypotheses.

The second hypothesis, namely that dinitrophenol catalyses the hydrolysis of an intermediate phosphate compound, was further developed by Lardy & Wellman (1952, 1953) and by Hunter (1951) and others (Green, 1951; Lee & Eiler, 1953; Cooper & Lehninger, 1957). In terms of Scheme I, this theory is that dinitrophenol promotes the hydrolysis of the ~P compound at site *B*. The ~P compound was thought of as an enzyme ~ phosphate compound which could react with dinitrophenol to give a dinitrophenol-enzyme compound which then decomposes liberating the free enzyme. In this way Lardy & Wellman (1953) explained the observation that dinitrophenol induces an ATPase (Hunter, 1951; Kielley & Kielley, 1951; Potter & Recknagel, 1951). We suggested a modification of this theory in which dinitrophenol reacted with A ~ C rather than with a ~P compound, i.e. at site *A* rather than site *B*.

The alternative theory of Lardy & Elvehjem (1945) for explaining the action of dinitrophenol, viz. that it allows oxidation to proceed without phosphorylation, has recently been developed in specific terms by Grabe (1958), Löw (1959a, b) and Löw *et al.* (1958). Their mechanism can be described by the equations

$$\mathrm{DPNH + OH^- + fp + P_i \rightleftharpoons DPN^+ + fpH \sim P}$$
$$\mathrm{fpH \sim P + 2Fe^{3+} + ADP \rightleftharpoons fp + 2Fe^{2+} + ATP + 2H^+}$$

Sum: $\mathrm{DPNH + 2Fe^{3+} + ADP + P_i \rightleftharpoons DPN^+ + 2Fe^{2+} + ATP + H^+}$

where $2Fe^{3+}$ and $2Fe^{2+}$ stand for the oxidized and reduced electron carrier, respectively, next to flavin in the cytochrome region. The dinitrophenol-induced ATPase is explained by the sequence

$$\mathrm{ATP + fpH_2 \rightleftharpoons ADP + fpH \sim P}$$
$$\mathrm{fpH \sim P + DPN^+ + OH^- \rightleftharpoons fp + P_i + DPNH}$$

followed by the non-phosphorylative reduction of the flavin by DPNH, a reaction which is supposed to be promoted by dinitrophenol

$$\mathrm{DPNH + fp + H^+ \xrightarrow{(dinitrophenol)} DPN^+ + fpH_2}$$

The sum of these last three reactions is

$$ATP + H_2O \xrightarrow{\text{(dinitrophenol)}} ADP + P_i$$

There are, then, three main theories of the mechanism of action of dinitrophenol:

(a) The original theory of Lardy, which proposes that dinitrophenol reacts with and promotes the hydrolysis of an intermediate high-energy phosphate compound.

(b) Our modification of Lardy's theory, which proposes that the site of action of dinitrophenol is on a high-energy compound formed before the intervention of inorganic phosphate.

(c) Lardy & Elvehjem's alternative theory, viz. that dinitrophenol "allows" oxidation to proceed without phosphorylation.

The experiments which have been carried out in an attempt to allow a choice between these three mechanisms will now be discussed under the following headings:

(1) Is phosphate necessary for uncoupling by dinitrophenol?

(2) Is there more than one dinitrophenol-induced ATPase?

(3) Is the dinitrophenol-induced ATPase affected by the state of oxidation and reduction of the respiratory chain?

Is phosphate necessary for uncoupling by dinitrophenol?

A clear-cut choice can be made between theory (a), on the one hand, and theories (b) and (c), on the other, by determining if inorganic phosphate is necessary for respiration in the presence of dinitrophenol. As explained above, phosphate is necessary as a catalyst if dinitrophenol reacts at site *B*, as in theory (a) but not if it reacts at site *A*. Phosphate is also not necessary in theory (c).

It should be remembered that there are three dinitrophenol-sensitive steps in the respiratory chain, and the mechanism for all three need not be the same. It is conceivable that one step would not be uncoupled, while the other two were. However, in that case respiration would still be blocked, because only one point of the respiratory chain need be blocked for complete inhibition of respiration.

The question which is posed as the heading of this section appeared to have been clearly answered in 1948 by Loomis & Lipmann, who found that dinitrophenol could replace the inorganic phosphate which was necessary for optimal oxidation rates with a kidney-cyclophorase preparation oxidizing glutamate. However, this conclusion was not supported by Teply in 1949 or by Judah in 1951, who found that if the mitochondria were pre-treated so as to remove endogenous inorganic phosphate, the maximum rates of oxidation were not obtained in the presence of dinitrophenol, unless phosphate was also added. Judah concluded that dinitrophenol split a phosphate-containing intermediate, a point of view which he has recently re-emphasized (Dawkins *et al.*, 1959).

This question has recently been re-examined in our laboratory (Borst & Slater, 1961). That fraction of the endogenous inorganic phosphate which is available to the mitochondrial respiratory chain was removed by a pre-incubation in a medium

containing substrate, ADP, glucose, hexokinase and magnesium. The main conclusions may be summarized as follows:

1. In agreement with Teply (1949) and Judah (1951), dinitrophenol* was found to stimulate the oxidation of glutamate to a much smaller extent than inorganic phosphate. However, this was found to be due to the fact, hitherto unsuspected, that the oxidation of glutamate by isolated mitochondria proceeds by a cyclic mechanism which involves the oxidation of α-ketoglutarate to succinate. It is known that the substrate-linked phosphorylation in this oxidation is not uncoupled by dinitrophenol (Hunter, 1951; Judah, 1951).

2. Succinate (in the presence of amytal) and proline are oxidized in the presence of dinitrophenol as rapidly as with phosphate.

3. Dinitrophenol stimulates the oxidation of malate or β-hydroxybutyrate, or of citrate in the presence of malonate, to a lesser degree than inorganic phosphate, but this is due to inhibition by dinitrophenol. The rate of oxidation in the presence of dinitrophenol is not further stimulated by the addition of phosphate.

The pre-incubation in the absence of added inorganic phosphate was found to reduce the level of inorganic phosphate from 100–150 μM† to 17 μM (or about 4 μmole P_i/g mitochondrial protein) with glutamate and 30 μM with β-hydroxybutyrate. The addition of dinitrophenol caused a slow increase in this level, presumably due to hydrolysis of the hexose monophosphate, dinitrophenol preventing the re-esterification of the inorganic phosphate set free. Since, however, the stimulation of respiration by dinitrophenol was maximal in less than 1 min, it was concluded that the inorganic phosphate set free played no role in the activation of the respiration by dinitrophenol.

These experiments have convinced us that phosphate is not necessary for the uncoupling of oxidative phosphorylation by dinitrophenol. Thus, theories (b) and (c) are preferred to theory (a).

Is there more than one dinitrophenol-induced ATPase?

The fact that all three phosphorylating steps of the respiratory chain are uncoupled by dinitrophenol can, in general, be explained in two ways:

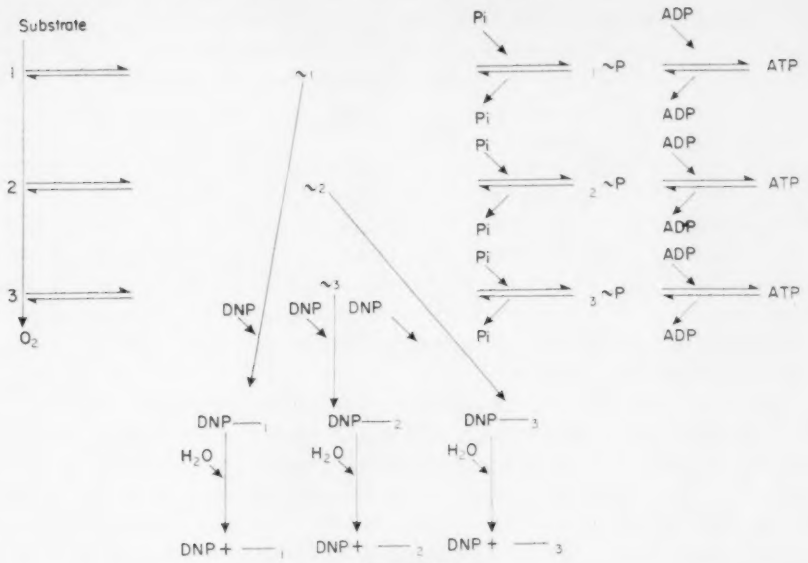
(1) The mechanisms of the three steps are rather similar, and the three ~ compounds formed by reaction (i) of Scheme I have similar chemical properties and can react with dinitrophenol according to Scheme II.

(2) The first ~ compound formed by the oxidation reaction is converted by a series of reactions to a common intermediate, which is capable of reacting with either dinitrophenol or inorganic phosphate, as in Scheme III.

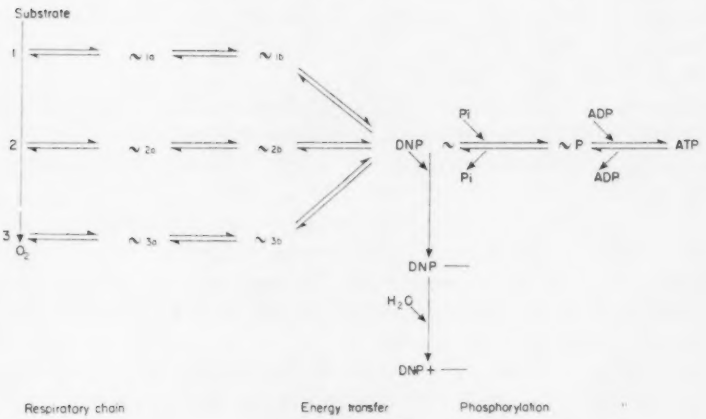
It should be noted that, if there is a common intermediate of oxidative phosphorylation according to this scheme, there must be several intermediate high-energy compounds. Clearly A~C (in reaction (1)) cannot be the common

* Similar results were obtained with *p*-nitrophenol, 4-*iso*amyl-2,6-dinitrophenol and gramicidin.

† These amounts of endogenous inorganic phosphate could be the reason why Loomis & Lipmann (1948) found that dinitrophenol was sufficient for maximum oxidation of glutamate.



SCHEME II.



SCHEME III.

intermediate since A is different at every step of the respiratory chain. There must, then, be a transfer of energy, thus

$$A\sim C+D\rightleftharpoons A+C\sim D^{*}.$$

This is represented by the reactions $\sim_{1a}\rightleftharpoons\sim_{1b}$, etc., in Scheme III. However it seems unlikely that the same C would be operative with different AH_2's and B's, of

* C corresponds to I and D to X in the mechanisms of Chance & Williams (1956) and Myers & Slater (1957), which fall in between the two extremes represented above by Schemes II and III.

widely different oxidation-reduction potential and chemical nature, in the three reactions (1). Thus, if there is to be only one dinitrophenol-sensitive compound, this cannot contain C, so that an additional energy transfer is involved

$$C \sim D + E \rightleftharpoons C + D \sim E.$$

This is represented by the reactions $\sim_{1b}\rightleftharpoons$ etc. in Scheme III. A similar scheme is implied in the mechanism proposed by Aldridge & Parker (1960).

Scheme III requires that there be only one dinitrophenol-induced ATPase. Scheme II and also the Stockholm mechanism (theory (c)) would lead one to expect three ATPases. However, the relative activities of the three would depend upon the ease of the reversibility of the various reactions (2) (i.e. reactions (ii) and (iii) in Scheme I), and it would be possible that one phosphorylative step quantitatively dominated the ATPase.

Myers & Slater (1957) in our laboratory found evidence from the pH-activity curve of the dinitrophenol-induced ATPase of rat liver mitochondria that there were three enzyme systems, characterized by pH optima at about 6·3, 7·4 and 8·5, respectively. These different enzymes had different sensitivities to dinitrophenol, the 6·3 enzyme being the most sensitive and the 8·5 enzyme the least (50 per cent maximum stimulation by 8, 18 and 60 μM dinitrophenol, respectively—see Slater & Hülsmann (1959)). Experiments with different concentrations of magnesium (Myers & Slater, 1957) and of ATP (Slater, 1957) excluded the possibility that the different peaks of activity were a reflection of the effect of pH on the concentration of the active form of ATP.

In our paper (Myers & Slater, 1957), we discussed the possibility that the decreasing activity of dinitrophenol with increasing pH might be due to the fact that the activator is the undissociated phenol, which decreases in concentration with increasing pH. We rejected this, because at pH 7·4 *p*-nitrophenol and 2,4-dinitrophenol have similar uncoupling activity, whereas the concentrations of the undissociated phenols differ by a factor of about 1000 at this pH (pK of 2,4-dinitrophenol, 4·1; of *p*-nitrophenol, 7·2). We concluded that the active form of dinitrophenol is the ion and that changes of pH between 5·5 and 10 affect the enzyme system rather than the inhibitor.

Our conclusion that the active form of uncoupling phenols is the ion was supported by Parker's finding (1958) that, at pH 6·8, the most potent uncouplers of oxidative phosphorylation in rat liver mitochondria are those phenols with the lowest pK's. Gladtke & Liss (1958), on the other hand, drew attention to cases where there was a poor correlation between the pK and the uncoupling activity. For example, 3,5-dinitrophenol and *p*-nitrophenol have about the same pK, but the former is 10 times as active as the latter; 2,4-dinitrophenol is only slightly more active than 3,5-dinitrophenol although its pK is much lower (4·0, compared with 6·7).

These authors (cf. De Deken, 1955) introduced a new factor into the consideration of the relative effectiveness of substituted phenols as uncoupling agents, namely the lipid solubility. Liss (1958) showed that the activity of

3,5-dinitrophenol in abolishing the Pasteur effect in yeast could be increased by an alkyl substituent in the 4-position, tenfold in the case of a *tert*butyl group. Similar although less pronounced results were obtained with respect to uncoupling of oxidative phosphorylation in liver mitochondria, e.g. 4 *tert*butyl-2,6-dinitrophenol was 7 times more active than 2,6-dinitrophenol (Gladtke & Liss, 1958).

Independently in our laboratory, Hülsmann (1958) also emphasized the importance of the liquid solubility of the uncoupling phenols. He found that 4-*iso*amyl-2,6-dinitrophenol was much more inhibitory than 2,6-dinitrophenol and concluded "that an uncoupler in high concentrations inhibits the respiration and that the concentration in the lipid mitochondrial membrane can be increased by the lipid solubility of the uncoupler" (translation of pp. 86–87).

Myers & Slater (1957), Parker (1958) and especially Hülsmann (1958) all draw attention to the inhibition of the dinitrophenol-induced ATPase by higher concentrations of dinitrophenol. The concentration (C_{opt}) giving maximum ATPase has been used by Hemker & Hülsmann (1961) in our laboratory for a quantitative study of the relationship between the solubility and the uncoupling activity of nitrophenol compounds.

Hemker's main findings may be summarized thus:

1. At a fixed pH, C_{opt} was found to decrease with increasing lipophilic character of the nitrophenol.

TABLE 1—COMPARISON OF OPTIMAL CONCENTRATIONS OF DIFFERENT NITROPHENOLS AT VARIOUS pH'S FOR STIMULATION OF ATPASE ACTIVITY OF RAT-LIVER MITOCHONDRIA

Compound	pK	pQ*	pC_{opt}†				
			pH 5	6	7	8	9
p-nitrophenol	7·2	+0·45	3·30	3·37	3·51	3·39	—
2,6-dinitrophenol	3·7	−2·35	4·77	4·22	3·68	3·12	—
2,6-dinitro-3,4-dimethylphenol	‡	‡	4·92	4·51	4·36	3·95	3·60
2,6-dinitro-4-*iso*butylphenol	4·3	−3·54	5·16	4·88	4·71	4·48	4·26
2,6-dinitro-4-*iso*amylphenol	4·1	−4·15	5·55	5·42	5·18	4·92	4·71
2,6-dinitro-4-*iso*-octylphenol	4·05	−4·75	6·08	5·89	5·64	5·41	5·24

Data of Hemker & Hülsmann (1961).

* $pQ = -\log_{10} \frac{\text{concentration of undissociated phenol in xylene}}{\text{concentration of undissociated phenol in water}}$. For the dinitrophenols at pH's >5, $pQ = pK - pH - \log_{10} \frac{\text{concentration of undissociated phenol in xylene}}{\text{concentration of total phenol in water}}$.

† $pC_{opt} = -\log_{10} C_{opt}$.

‡ $pK - pQ = 7{\cdot}44$.

2. For the series of 2,6-dinitrophenol compounds shown in Table 1, the ATPase activities at C_{opt} for a particular compound were the same, amounting to 75 per cent of the value reached with *p*-nitrophenol or 2,4-dinitrophenol.

3. For each of the nitrophenols, except *p*-nitrophenol, C_{opt} increased with increasing pH. A linear relationship was found between pH and pC_{opt} ($-\log_{10} C_{opt}$), which could be expressed by the equation

$$pC_{opt} = a - b\,pH$$

b decreased with increasing lipophilic character (from 0·56 for both 2,4- and 2,6-dinitrophenol to 0·20 for the *p-iso*-octyl compound).

4. From the above it follows that the pH-activity curve of the dinitrophenol-induced ATPase at a fixed dinitrophenol concentration has little meaning. It is necessary to determine the relationship between pH and the maximum activity measured at the C_{opt} corresponding to each pH. When this was done, a maximum at pH 6·9 was found for 2,4-dinitrophenol.

5. *p*-Nitrophenol differs from the dinitrophenols studied by virtue of its much higher pK and lower lipid solubility (see Table 1). With this compound pC_{opt} was found to be largely independent of pH between pH's 5 and 7. Above pH 8, it followed the same relationship as given above for the dinitrophenols.

Assuming that

(i) the undissociated phenol must be taken up by the mitochondrial lipid before it uncouples oxidative phosphorylation,

(ii) the partition coefficient between mitochondrial lipid and water is the same as that between xylene and water,

(iii) the maximum ATPase is obtained with the same concentration in the mitochondrial lipid (C_L) of the 2,6-dinitrophenols independent of the size of the lipophilic substituents or of the pH, Hemker was able to show that, at pH's greater than pK,

$$pC_L = pC_{opt} - pK + pH + pQ.$$

In agreement with this prediction, Table 2 shows that, at constant pH, pC_L is independent of the degree of the lipophilicity of the 4-substituted 2,6-dinitrophenols. However, pC_L calculated from this expression increased with increasing pH, according to the equation $pC_L = -1{\cdot}49 + 0{\cdot}76$ pH, i.e. C_L necessary for maximal ATPase is less the higher the pH.

Hemker's results do not prove that it is the undissociated phenol which reacts with the ~ compound in the mitochondrial lipid. What is clear, however, is that the concentration of the undissociated phenol in the medium determines the degree of uncoupling by a particular dinitrophenol and that this is the explanation of the decreasing degree of uncoupling with increasing pH. In comparing different nitrophenols, two factors have to be considered—the pK and the lipid solubility. It is the latter which was overlooked in our previous work (Myers & Slater, 1957). In a series of dinitrophenols which all have about the same pK, it is the lipid solubility which determines the relative degree of uncoupling. When comparing *p*-nitrophenol with 2,4-dinitrophenol the difference in pK (3·1) is largely compensated in the other direction by the difference in pQ (2·55), so that the fact

that around neutral pH the two compounds uncouple to about the same extent is purely coincidental.

TABLE 2—CALCULATION FROM DATA IN TABLE 1 OF CONCENTRATION IN MITOCHONDRIAL LIPID (C_L) OF DIFFERENT NITROPHENOLS AT VARIOUS pH's

$$pC_L = pC_{opt} - pK + pQ + pH$$

Compound	pK	pQ	pC_L				
			pH 5	6	7	8	9
p-nitrophenol*	7·2	+0·45	3·55	3·62	4·06	4·62	—
2,4-dinitrophenol	4·1	−2·06	3·61†	4·06†	4·52†	4·96†	—
2,6-dinitrophenol	3·7	−2·35	3·72	4·17	4·63	5·07	—
2,6-dinitro-3,4-dimethylphenol	‡	‡	2·48	3·07	3·82	4·51	5·16
2,6-dinitro-4-*iso*butylphenol	4·3	−3·54	2·33	3·05	3·88	4·65	5·43
2,6-dinitro-4-*iso*amylphenol	4·1	−4·15	2·30	3·04	3·93	4·67	5·56
2,6-dinitro-4-*iso*-octylphenol	4·05	−4·75	2·28	3·09	3·84	4·61	5·44

* In this case, the simple expression pC_I is not applicable, since the pH is not greater than pK. pC_I was calculated from the expression $pC_I = pC_{opt} + pQ + pH + \log (K + (H^+))$.

† This compound was not so extensively investigated as the 2,6-dinitrophenol, but no significant difference was found between the values of pC_{opt} for the two compounds. For purposes of calculation, it has been assumed that they were identical.

‡ $pK - pQ = 7{\cdot}44$.

It must be concluded from Hemker's studies that it is not possible to identify more than one dinitrophenol-induced ATPase on the basis of pH-activity curves.§ There remain the following possibilities:

1. Scheme II is operating, and

(a) there are three dinitrophenol-induced ATPases with rather similar pH optima and sensitivity to dinitrophenol; or

(b) one of phosphorylative steps quantitatively dominates the ATPase reaction.

§ It should be noted that this conclusion does not invalidate the evidence that the Mg^{2+}-stimulated ATPase of disrupted mitochondria is multiple (Myers & Slater, 1957) nor that indicating that the different phosphorylating steps in oxidative phosphorylation have different pH optima (Hülsmann & Slater, 1957; Hülsmann, 1958). Mechanisms of the type (cf. Slater, 1958; Hülsmann, 1958)

$$\begin{aligned} ATP + I &\rightarrow \sim I + ADP + P_i \\ \sim I + Y &\rightarrow I - Y \\ I - Y + H_2O \rightleftharpoons I + Y \text{ or } I - Y + H_2O &\rightarrow I + Y \\ I - Y + Y &\rightleftharpoons I - Y_2 \end{aligned}$$

where I is an intermediate and Y is the uncoupling phenol, predict that $dpC_L/dpH = 0{\cdot}5$ under two possible circumstances: (i) when Y is the undissociated phenol and I − Y is hydrolysed by H^+ ions; (ii) when Y is the phenolate ion and I − Y is hydrolysed by OH^- ions.

2. Scheme III is operating.

Penniall (1960) has found that soluble (i.e. not sedimented in 60 min at 105,000 × g) extracts of mitochondrial acetone powders in 0·25 M sucrose exhibit multiphase curves of ATPase response to dinitrophenol at pH 8·5, when tested in the absence of added salts. Peaks of response occurred at 10^{-8}, 10^{-5} and 10^{-3} M, respectively.

It is difficult to discuss the published Stockholm mechanism in terms of Hemker's results, since the action of dinitrophenol has not been formulated in precise chemical terms in this mechanism.*

Is the dinitrophenol-induced ATPase affected by the state of oxidation and reduction of the respiratory chain?

It has been shown above that studies of the dinitrophenol-induced ATPase at different pH's have not enabled a choice to be made between the two versions of our mechanism given in Schemes II and III, or indeed between this mechanism and the Stockholm.

Another experimental approach is to determine if the dinitrophenol-induced ATPase is affected by the state of oxidation and reduction of the respiratory chain.† According to Scheme II, in its simplest form, the dinitrophenol-sensitive ~ compounds are written as A ~ C (see reaction (1)), i.e. each involves a component of the respiratory chain. If, then, A is kept fully reduced, e.g. by adding substrate in the absence of oxygen or in the presence of cyanide, it would be expected that the ATPase reaction would be fully inhibited. This would not be expected from a mechanism such as Scheme III (or even one which involved dinitrophenol-induced hydrolysis of $\sim_{1b}$, $\sim_{2b}$ and $\sim_{3b}$), where the dinitrophenol-sensitive compound does not contain A or BH_2.

The Stockholm mechanism requires reduced flavin and DPN^+ for the ATP-requiring step, and DPNH and oxidized flavin for the dinitrophenol-promoted reaction. In fact, it is known that, in the absence of added substrate and in the presence of dinitrophenol, components of the respiratory chain are very largely in the oxidized state (Chance & Williams, 1956). One might question whether there would be sufficient reduced flavin under these conditions to bring about reduction of the DPN^+ in the presence of ATP. Reduction of the respiratory chain would be expected to favour this reaction, and thereby promote the dinitrophenol-induced ATPase provided that it does not proceed so far as completely to reduce the flavin.

* Ernster & Grabe (1960) have recently suggested that in intact mitochondria no H^+ ions are present, so that the oxidation of DPNH, which requires an H^+ ion, cannot occur, except in the presence of phosphate or in the presence of dinitrophenol which can replace phosphate by facilitating the uptake of H^+ ions from water. It would be interesting to calculate whether Hemker's data are consistent with this suggestion.

† The reader is referred to the introduction of a paper by Vignais & Vignais (1960) for a lucid discussion of how the ATPase reaction will depend upon the state of oxidation of the respiratory carriers, on the basis of the various mechanisms of oxidative phosphorylation which have been proposed.

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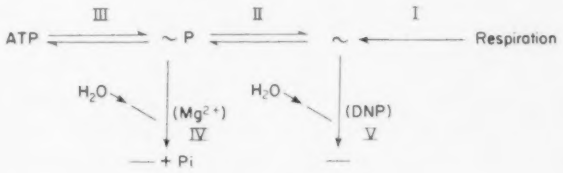
Before discussing the literature on the effect of the state of oxidation-reduction of the respiratory chain on the dinitrophenol-induced ATPase, it is necessary to make a clear distinction between this ATPase reaction and the so-called Mg^{2+}-stimulated ATPase, and to discuss the relationship between these reactions and two exchange reactions catalysed by mitochondria, viz. between P_i and ATP, and between ADP and ATP. Freshly prepared rat liver mitochondria have little if any ATPase activity in the absence of added dinitrophenol, even when Mg^{2+} is added. Since the formation of the ~ compound from ATP (see Scheme I) proceeds through the ~ P compound, it follows that the latter is not hydrolysed. However, structural damage to the mitochondria, such as that brought about by aging followed by freezing and thawing (see Myers & Slater (1957) which includes a discussion of the earlier literature) or by the addition of deoxycholate (Siekevitz *et al.*, 1958) brings about the development of a Mg^{2+}-requiring ATPase, with a corresponding decrease in the degree of stimulation of the ATPase (measured in the presence of added Mg^{2+}) by the addition of dinitrophenol. These findings can be explained by the hypothesis that structural damage of this nature makes the ~ P compound accessible to water* so that it is hydrolysed in a Mg^{2+}-requiring reaction.

We can express this in terms of Scheme I by the reaction sequence (cf. Purvis & Slater, 1959)



According to this explanation, which is essentially the same as that given by Siekevitz *et al.* (1958) (cf. also Hunter, 1955), the Mg^{2+}-stimulated and the dinitrophenol-induced ATPases differ in the nature of their hydrolytic reaction, but both contain the same phosphorylation reaction (iii), which is moreover the same reaction as in oxidative phosphorylation, but in the opposite direction. In completely disrupted mitochondria, the ~ P compound is so readily hydrolysed that, in the presence of added Mg^{2+}, the ~ compound is never formed from ATP. Thus, under these conditions there will be no stimulation of the ATPase by dinitrophenol. In intact mitochondria, the ~ compound is stable even in the presence of Mg^{2+}, so that there is no ATPase activity in the absence of added dinitrophenol. Heart sarcosomes form an intermediate situation, i.e. there is little ATPase activity (except at high pH) in the absence of dinitrophenol or of added Mg^{2+}, while the activity is partially stimulated by Mg^{2+} and fully by the addition

* For the purposes of the present discussion, it is not necessary to go into the question whether this is purely a physical phenomenon or is caused by the liberation of unsaturated fatty acids (Hülsmann, 1958).

of both Mg^{2+} and dinitrophenol (Holton *et al.*, 1957). The results of Cooper & Lehninger (1957) and Cooper (1958) can be explained on the same basis. Löw (1959a, b) proposed that the ~ P compound hydrolysed by reaction (iv) is a reduced flavin phosphate compound.

The various reactions related to oxidative phosphorylation which are catalysed by mitochondria involve different reactions of the above scheme, as shown in Table 3.

TABLE 3—REACTIONS INVOLVED IN MITOCHONDRIAL RESPIRATION, ATPASES AND EXCHANGE REACTIONS

(+) indicates that the reaction is involved, (−) that this reaction will cause an inhibition of the exchange measured.

	Reaction				
	(i)	(ii)	(iii)	(iv)	(v)
Respiration	+	+	+		
	+	+		+	
	+				+
DNP-induced ATPase		+	+		+
Mg^{2+}-stimulated ATPase			+	+	
$P_i \rightleftharpoons$ ATP exchange reaction		+	+	−	−
ADP $\rightleftharpoons$ ATP exchange reaction			+	−	−*

* When reaction (ii) is intact.

It should be noted that, although both the P_i–ATP exchange reaction and the dinitrophenol-induced ATPase involve reactions (ii) and (iii), there is an important difference between the conditions of the two tests. The presence of dinitrophenol in the measurement of the ATPase leads to a lower steady-state concentration of the high-energy compounds in this test than in the exchange reaction. Consequently, there will be more opportunity during the measurement of the exchange reaction for side reactions involving the high-energy compounds. A second possible complication in the exchange reaction is that respiration (reaction (i)) caused by the presence of endogenous substrates can increase the rate of formation of the ~ compound, and thereby contribute to the rate of the exchange reaction.

Effect of inhibitors of the respiratory chain. Myers & Slater (1957) reported that the addition of 1 mM KCN to rat liver mitochondria in the presence of 0·1 mM dinitrophenol had no effect on the ATPase. Since endogenous substrate would be expected to reduce the entire chain in the presence of KCN, while dinitrophenol in the absence of KCN causes the rapid exhaustion of endogenous substrate and oxidation of all components of the chain (Chance & Williams, 1956) it was concluded that the state of oxidation and reduction of the respiratory chain had no effect on the dinitrophenol-induced ATPase of liver mitochondria. Similarly, the addition of 1 mM KCN and succinate had no effect on the Mg^{2+}-stimulated ATPase of mitochondrial fragments.

This conclusion was supported by Cooper & Lehninger (1957) working with "digitonin particles" and by Löw (1959b) with intact mitochondria, and was also in agreement with a number of observations on the closely related ATP–P_i exchange reaction (Cooper & Lehninger, 1957; Swanson, 1956; Boyer *et al.*, 1956; Hülsmann *et al.*, 1958).

Other reports including the most recent, however, bring forward evidence that either the addition of cyanide, or anaerobiosis, causes the inhibition of both the dinitrophenol-induced ATPase and the ATP–P_i exchange reaction, without having any effect on the Mg^{2+}-stimulated ATPase in mitochondrial fragments or on the ADP–ATP exchange reaction.

Hunter (1955) reported an average inhibition of 35 per cent of the dinitrophenol-induced ATPase brought about by anaerobiosis. Boyer *et al.* (1956) found that, although 0·1 mM KCN, sufficient for nearly complete inhibition of respiration, had little effect even in the presence of substrate on the P_i–ATP exchange reaction, higher concentrations produced some inhibition which did not, however, amount to more than about 30 per cent even with 10 mM KCN.

Wadkins & Lehninger (1957, 1959); Lehninger *et al.* (1958) made a detailed study of the effect of 1–2 mM cyanide or anaerobiosis on the ATPase and exchange reactions. Using digitonin particles they found 68–85 per cent inhibition of the P_i–ATP exchange reaction by anaerobiosis in the presence of β-hydroxybutyrate, and 65–90 per cent inhibition by cyanide + β-hydroxybutyrate. Succinate could replace β-hydroxybutyrate, and the greatest inhibition was found with the two substrates together. The dinitrophenol-induced ATPase was inhibited 76 per cent by β-hydroxybutyrate + succinate, in the presence of cyanide, maximum inhibition being found at pH 7·5. There was only a 15 per cent inhibition of the Mg^{2+}-stimulated ATPase under the same conditions and no effect on the ADP–ATP exchange.

With intact rat liver mitochondria, Wadkins & Lehninger (1959) found up to 90 per cent inhibition of the P_i–ATP exchange reaction by β-hydroxybutryate, in the presence of cyanide or the absence of oxygen, but, in agreement with Myers & Slater (1957), they found no effect on the dinitrophenol-induced ATPase.

Vignais & Vignais (1960) also found some inhibition of the P_i–ATP exchange reaction of digitonin particles by anaerobiosis or addition of cyanide.

In our laboratory, Chefurka (1960) studied the effect on the ATPase reaction and on the P_i–ATP exchange reaction of: (i) high concentrations (about 10 mM) cyanide in the absence of added substrate; (ii) lower concentrations (0·1 mM) cyanide in the presence of substrate; and (iii) complete anaerobiosis. The dinitrophenol-induced ATPase of rat liver mitochondria was inhibited by about 60–75 per cent by all treatments, while anaerobiosis inhibited the exchange reaction 30–40 per cent. Complete reversibility of the inhibition of the ATPase by anaerobiosis could be demonstrated. These treatments had no effect on the Mg^{2+}-stimulated ATPase in mitochondrial fragments, or in heart sarcosomes, while the dinitrophenol-induced ATPase of the sarcosomes was affected in the same way (although to a smaller extent) as in rat liver mitochondria.

Maximum inhibition of the dinitrophenol-induced ATPase in rat liver mitochondria was obtained at pH 7·2. The residual activity was little dependent upon pH between 5·6 and 9·2.

The most direct interpretation of Wadkins & Lehninger's and Chefurka's experiments is that reduction of the respiratory chain, caused by any one of the three treatments used, brings about a decrease of the concentration of the dinitrophenol-sensitive ~ compound, with a consequent decline in the rate of the dinitrophenol-induced ATPase and in the exchange reaction. This would be understandable if this ~ compound (A ~ C) involves a component of the respiratory chain in its oxidized form (A) as in the original version of our formulation (1953). The lack of sensitivity of the Mg^{2+}-stimulated ATPase or the ADP–ATP exchange would be understandable if the ~P compound involved C (C ~ P) rather than A (cf. Wadkins & Lehninger, 1959; Chefurka, 1960).

Chefurka (1960) suggests that the anaerobiosis- and cyanide-resistant dinitrophenol-induced ATPase is due to reaction of dinitrophenol with the ~ P compound. Such a reaction might also be the explanation of the partial stimulation by dinitrophenol of the soluble ATPase isolated from beefheart mitochondria by Pullman *et al.* (1960; Penefsky *et al.*, 1960).

If these interpretations are correct, Scheme II is favoured over Scheme III, since the dinitrophenol-sensitive ~ compound is closer to the respiratory chain. As already discussed, reduction of the respiratory chain might be expected to increase rather than decrease the dinitrophenol-induced ATPase, on the basis of the Stockholm mechanism.* The lack of an effect of reduction on the Mg^{2+}-stimulated ATPase would appear to be decisive evidence against Löw's explanation of this reaction, which requires reduced flavin.

It would probably be wise, however, to be cautious in interpreting these experiments. The many reports in the literature, prior to the studies of Wadkins & Lehninger and of Chefurka, suggesting that reduction of the chain had no effect on the ATPase or on the exchange reaction, give reason for suspecting that the systems are not fully understood. Moreover, Chefurka has drawn attention to a number of puzzling features, e.g. (i) concentrations of cyanide (0·1 mM) sufficient to inhibit mitochondrial respiration almost completely have no effect, unless substrate is also added; (ii) the inhibition by high concentrations of cyanide virtually disappeared at higher concentrations of mitochondria; (iii) in the presence of 0·1 mM cyanide, succinate was much more effective in inhibiting the ATPase than glutamate or β-hydroxybutyrate. It is difficult to relate this to the succinate-induced reduction of pyridine nucleotide (Chance & Hollunger, 1960), since the latter does not take place in the presence of dinitrophenol. In fact, the whole question of reduction of the mitochondrial respiratory chain in the presence of dinitrophenol is poorly

* As Wadkins & Lehninger (1959) have pointed out, the Stockholm mechanism would require that the rate of P_i–ATP exchange is minimal in the fully oxidized and fully reduced states and maximal in intermediate states. These authors could find no evidence that ATPase and exchange activity was maximal at intermediate states, obtained by adding increasing concentrations of cyanide.

understood (*Intern. Symp. on Biological Structure and Function*, 1960). The incomplete inhibition by cyanide or anaerobiosis might be due to incomplete reduction of the respiratory-chain component concerned (see Wadkins & Lehninger (1959) for a discussion of other possibilities).

It should also be mentioned that the inhibition of the P_i–ATP exchange reaction was only found by Wadkins & Lehninger under certain circumstances, viz. at very low P_i and ADP concentrations.

If it can be tentatively accepted that reduction of the respiratory chain causes inhibition of the dinitrophenol-induced ATPase and the P_i–ATP exchange, it is clearly of importance to try to locate the region of the chain involved by the use of specific inhibitors, e.g. amytal which causes reduction of DPN^+ and oxidation of the rest of the chain, and antimycin which causes reduction of DPN^+, flavoprotein and cytochrome *b* and oxidation of the remainder of the chain.

Unfortunately, the effects of these inhibitors are difficult to interpret, since both cause appreciable stimulation of the ATPase in the absence of dinitrophenol (Myers & Slater, 1957; Hemker & Hülsmann, 1961). This causes a special difficulty in the interpretation of the effects of these inhibitors on the P_i–ATP exchange reaction, since any uncoupler, whether it promotes reaction (iv) or (v), will inhibit the exchange reaction, whatever it does to the state of oxidation-reduction of the respiratory chain.

Amytal has no effect on the Mg^{2+}-stimulated ATPase (Siekevitz *et al.*, 1958). Aldridge & Parker (1960) found no inhibition of the dinitrophenol-induced ATPase with 1 mM amytal, which was 4 times the concentration necessary completely to inhibit respiration in their experiments. Siekevitz *et al.* found only a 10 per cent inhibition at this concentration, but reported increasing inhibition at greater concentrations. High concentrations also inhibit the exchange reaction.

Siekevitz *et al.* found no effect of antimycin on either the dinitrophenol-induced or on the Mg^{2+}-stimulated ATPase, although there is some inhibition of the P_i–ATP exchange reaction (Löw *et al.*, 1958; Hülsmann, 1958).

The author considers that further study of the effects of these two inhibitors would be rewarding. It is noteworthy that although they stimulate the ATPase, they have little effect on the P : O ratios of those portions of the respiratory chain which they do not inhibit (Ernster *et al.*, 1955; Slater, 1955).

Fluoride inhibits the dinitrophenol-induced ATPase much more than the Mg^{2+}-stimulated (Myers & Slater, 1957; Siekevitz *et al.*, 1958), suggesting that it reacts with the $\sim$ compound. Inhibition of the ATPase reaction is by no means complete, so that the relatively slight effect of fluoride on respiration is not necessarily inconsistent with this interpretation.

Azide, oligomycin and trialkyltin inhibit both ATPases and the P_i–ATP exchange reaction, but have no effect on the ADP–ATP exchange. This cannot be explained on the basis of the schemes given above, since, according to these, the ADP–ATP exchange reaction is catalysed by reaction (iii) which is also involved in the Mg^{2+}-stimulated ATPase. It appears necessary to split reaction (iii) into two (as already suggested by Lardy (1960) for another reason) as is shown in Scheme IV.

Reaction (IIIa) catalyses the exchange reaction, while $\sim P_b$ is the site of action of these inhibitors and possibly also of guanidine.

OTHER INHIBITORS OF THE UNCOUPLING ACTION OF DINITROPHENOL

Table 4 summarizes the effects of a number of compounds on the uncoupling action of dinitrophenol on oxidative phosphorylation and on related reactions.

TABLE 4—EFFECTS OF INHIBITORS ON REACTIONS CATALYSED BY MITOCHONDRIA RELATED TO OXIDATIVE PHOSPHORYLATION

	Guanidine	Azide	Oligomycin	Trialkyltin	Fluoride
Respiration					
Mitochondria + ADP, P_i	+‡	?*	+‡	+	−
Mitochondria + DNP	−	?*	−	+	−
Mitochondrial fragments	−	?*	−	−†	−
ATP*ase*					
Dinitrophenol-induced	±	+	+	+	+
Mg^{2+}-stimulated	+	+	+	+	−
Exchange reactions					
$P_i \rightleftharpoons ATP$	?	+	+	+	?
$ADP \rightleftharpoons ATP$	?	−	−	−	?

(+) indicates inhibition, (−) no inhibition, (?) no information, (±) conflicting information.

* Cannot be studied because of action on cytochrome a_3.

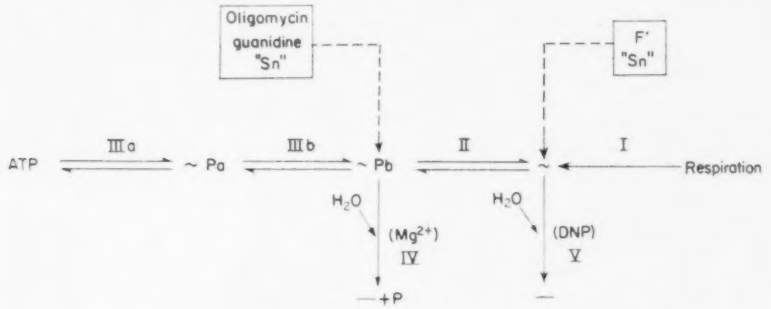
† Inhibited by higher concentrations.

‡ Little, if any, inhibition of the succinic oxidase system.

Guanidine and oligomycin, studied by Hollunger (1955) and Lardy (1958, 1959) respectively, are especially interesting in this respect, since the inhibition of the respiration which they bring about is relieved by dinitrophenol. This would be expected if these inhibitors act only on $\sim P_b$. The trialkyltins studied by Aldridge (1958, 1961) appear to be less selective in their action, since the inhibition is not relieved by dinitrophenol. Perhaps these compounds also combine with the $\sim$ compound. Oligomycin and guanidine have no effect on the respiration of mitochondrial fragments which would be understandable if the $\sim$ compound can be hydrolysed in these fragments even in the absence of dinitrophenol. Several explanations are possible for the relative resistance of the respiration of fragments to trialkyltin.

The oxidation of succinate by mitochondria is only partially (67 per cent) inhibited by oligomycin (Lardy *et al.*, 1958). Since a considerable proportion of the O_2 uptake measured with liver mitochondria oxidizing succinate is due to oxidation past malate (Greengard *et al.*, 1959), it seems possible that the oxidation of succinate to fumarate is completely insensitive to oligomycin. If this is the case, it would follow that the ATPase and exchange reactions studied in mitochondria are associated with only one of the phosphorylation steps. However, the fact that the phosphorylation associated with succinate oxidation is uncoupled is a complication.

The solution of this dilemma might be expected significantly to deepen our knowledge of the mechanism of oxidative phosphorylation.



SCHEME IV.

The earlier studied guanidine (Hollunger, 1955) behaves rather similarly to oligomycin, but much higher concentrations are required. Two reports (Hollunger, 1955; Myers & Slater, 1957) agree that the dinitrophenol-induced ATPase is less sensitive than the Mg^{2+}-stimulated, which is surprising, while, according to another report (Siekevitz *et al.*, 1958), there is no difference. Hollunger found that dinitrophenol, but not arsenate, reverses the inhibition by guanidine of the respiration. The lack of an effect of arsenate is somewhat puzzling, since there is evidence (Wadkins, 1960; Borst & Slater, 1961) that it reacts on the same ~ compound as dinitrophenol.

CONCLUSIONS

It is possible to give a definite answer to only one of the three questions posed in the Introduction, viz. is phosphate necessary for uncoupling by dinitrophenol?—which can be answered in the negative. This shows that nitrophenols operate between the respiratory chain and the intervention of inorganic phosphate.

The second question—is there more than one dinitrophenol-induced ATPase?—cannot be answered except with a weak "probably".

The third question—is the dinitrophenol-induced ATPase affected by the state of oxidation and reduction of the respiratory chain?—can be answered with a stronger "probably".

The experiments discussed in this paper can be adequately explained by Scheme II, in which each phosphorylative step can be described by the equations

$$AH_2 + B + C \rightleftharpoons A \sim C + BH_2$$
$$A \sim C + P_i \rightleftharpoons C \sim P + A$$
$$C \sim P + ADP \rightleftharpoons C + ATP$$
$$A \sim C + DNP \rightarrow A + C{-}DNP$$
$$C{-}DNP + H_2O \rightleftharpoons C + DNP$$

in which AH_2, B and C are different for each step.

As already explained elsewhere (Greengard, Minnaert, Slater & Betel, 1959; Slater, 1959), however, it is probable that the real mechanism is much more complicated, perhaps combining features of the simplified mechanism favoured by us (Slater, 1953, 1958) and the Stockholm mechanism (Löw, Siekevitz, Ernster & Lindberg, 1958; Grabe, 1958; Löw, 1959a, b).

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ENZYMES AND ENZYME MODELS

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THE determination of mechanisms of enzyme action is among the great problems of modern chemistry (Boyer *et al.*, 1960; Boyer, 1960; Westheimer, 1959). Although not one enzymatic process is today understood, and although no model system has achieved catalytic activity comparable to that of the corresponding enzyme, considerable progress has been made in elucidating the "active sites" in enzymes, and in finding catalytic systems which will operate (although slowly) under experimental conditions which are comparable to those in cells and, hopefully, by mechanisms which parallel the biochemical ones. The problem is under attack from many angles: studies of peptide sequences, X-ray analysis of protein structure, determination of the coenzyme or active sites needed for reaction, kinetic studies of enzyme action, and chemical "models" for various enzymatic systems. All of these methods provide information relevant to the central problem; how does a protein strongly accelerate a chemical reaction between a substrate and either a coenzyme molecule or an active site on the protein itself or some other reagent.

In subsequent sections of this review, some individual enzymes are considered in some detail. Before considering these systems, a few words might help set the problem in perspective. Much of our understanding of catalytic action has been gained by studies of model systems. The pioneers in such investigations (Langenbeck, 1935; Warburg 1949) did not have the present-day background of theoretical chemistry on which to build. In 1947, in a paper more pertinent to modern considerations of detailed mechanism, Braunstein suggested the outlines of a mechanism for transamination: this mechanism and the role of pyridoxal have been strongly substantiated (Braunstein, 1960; Metzler *et al.*, 1954; Ikawa & Snell, 1954) and are generally accepted today. More recently, the role of thiamin in the decarboxylation of pyruvate (Breslow, 1958), the action of diphosphopyridine nucleotides in the direct and stereospecific transfer of hydrogen (Vennesland & Westheimer, 1954), and the role of the serine residue in the reactions of esterases (Desnuelle, 1960a, b; Cohen *et al.*, 1959) and of phosphoglucomutase (Koshland & Erwin, 1957) have been discovered, and imitated with model systems. Some of these systems are here considered. The role of biotin and of several other coenzymes in enzymatic processes will be discussed in another session in this Congress. In a recent summary (Westheimer, 1959), the author stated, " . . . hopefully, in another decade, reviews may be concerned with enzymes and enzyme analogs (rather than with models) . . . ". The decade has not yet passed, and the prediction still seems reasonable.

RIBONUCLEASE

Structure

Ribonuclease is distinguished as the first enzyme for which the entire amino-acid sequence and the location of the disulfide bonds are known. The signal achievement of determining this structure is largely the work of Moore at the Rockefeller Institute (Hirs *et al.*, 1960; Hirs, 1960; Spackman *et al.*, 1960). Nevertheless, the mechanism of action of this enzyme is probably less well understood than that of others where structural information is less complete. Furthermore, although the sequence of amino-acids for this enzyme is known, the three-dimensional configuration may only be guessed. Neither the extent of α-helix nor the interactions of various helical and randomly coiled sections may be specified with assurance. The observed changes in optical rotation on opening the disulfide bonds suggest (Perlmann & Diringer, 1960) that the net contribution of right-handed helix (i.e. the excess of right- over left-handed helix) is small, and probably only of the order of 20 per cent. (Although it is generally assumed that right-handed helices predominate in proteins, the balance must be a delicate one. In synthetic polypeptides, poly-L-benzyl glutamate forms a right-handed helix, but poly-L-benzyl aspartate a left-handed one (Blout & Karlson, 1958; Bradbury *et al.*, 1959).) Alternate estimates of the helical content of ribonuclease, obtained from infra-red measurements and from the rapidity of hydrogen–deuterium exchange (Perlmann & Diringer, 1960; Scheraga, 1960) suggest a total of about 50 per cent of helical sections in the enzyme.

Chemical intermediates

The enzyme catalyzes the cleavage of ribonucleic acid at the phosphorus to oxygen bond connecting the phosphate ester group of a pyrimidine nucleotide at 3′- to the 5′-position in the ribose residue of the adjacent nucleotide. The enzymatic reaction almost certainly proceeds in two steps (Markham & Smith, 1952; Brown *et al.*, 1952; Richards & Vithayathil, 1959); first, the formation of a 2′, 3′-cyclic nucleotide, with cleavage of the P–O bond to the 5′-position of the adjacent residue, and second, the opening of the cyclic phosphate.

Non-enzymatic model systems

The non-enzymatic hydrolysis of dialkyl phosphates takes place very slowly. For example, the hydrolysis of dimethyl phosphate has a half-time of a day at 125° in 1 N alkali (Kumamoto *et al.*, 1956), and a half-time of 5 hr at 100° in 5 M perchloric acid solution (Bunton *et al.*, 1960). Furthermore, under both sets of experimental conditions, the hydrolysis occurs largely at the carbon to oxygen rather than at the phosphorus to oxygen bonds (Bunton *et al.*, 1960). On the other hand, phosphate esters which hold an hydroxyl group adjacent to the P–O linkage hydrolyze comparatively readily (Bailly, 1938; Verkade *et al.*, 1940; Chargaff, 1942) and presumably at the P–O bond. Considerable evidence shows that, in these instances, a five-membered cyclic phosphate is an intermediate: the reaction then consists of an internal displacement by the alcoholic group on phosphorus, which

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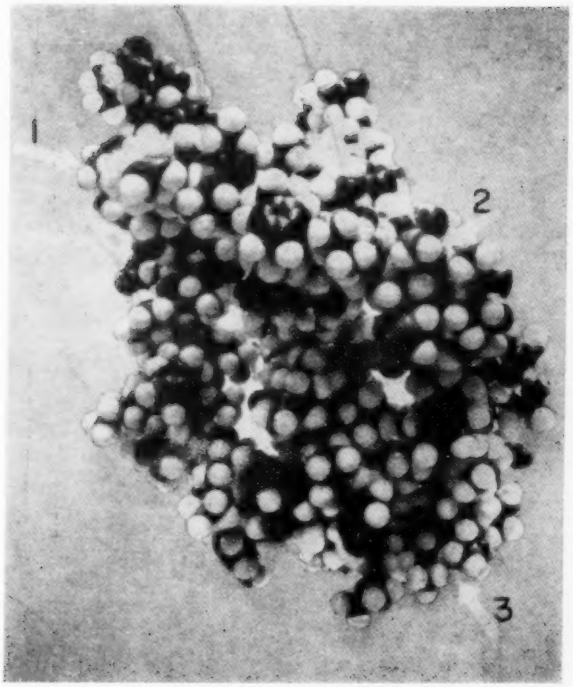

FIG. 1. The three-dimensional structure of ribonuclease as postulated by Scheraga and constructed with Pauling–Corey models.

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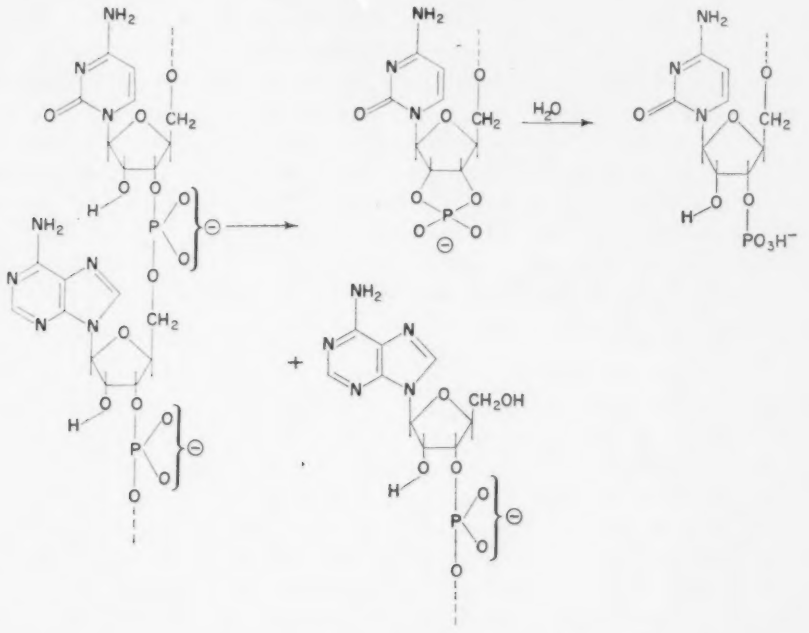
is highly favored over the corresponding external displacement by water or hydroxide ion (Brown *et al.*, 1956). The five-membered cyclic phosphate so formed is rapidly hydrolyzed: the opening of potassium ethylene phosphate in alkali



(Kumamoto *et al.*, 1956) proceeds about 10^7 times as fast as does the hydrolysis of potassium dimethyl phosphate, and about 10^6 times as fast as does that of potassium trimethylene phosphate (Khorana *et al.*, 1957). Similarly, the alkaline hydrolysis of methyl ethylene phosphate proceeds about 10^6 times as fast as does that of trimethyl phosphate, and about 1000 times as fast as does that of dimethyl hydroxyethyl phosphate (Wall, unpublished). Relative to the trialkyl ester, the hydroxyester hydrolyzes very rapidly, just as ribonucleic acid hydrolyzes more readily than does deoxyribonucleic acid. The high rates of the hydrolysis of the cyclic as compared to those of the non-cyclic esters invite speculation as to the origin of the large difference in rate. Presumably the proximate cause of the rapid reaction is strain in the five-membered ring; the ultimate reason for this strain is still unknown (Haake, 1960). This strain has, however, been measured thermochemically with methyl ethylene phosphate, and amounts to about 7–8 kcal/mole (Cox *et al.*, 1959). The comparative rates cited above suggest that the reactions catalyzed by ribonuclease are those which occur rather readily non-enzymatically (Westheimer, 1957; Cohn, 1959).

How fast, then, is the enzymatic reaction relative to the corresponding non-enzymatic processes? The data on which a firm comparison could be made are

not at hand. The second-order rate constant for the alkaline hydrolysis of potassium ethylene phosphate (Kumamoto *et al.*, 1956) is known and the reaction proceeds exclusively with P–O cleavage (Haake, 1960). The rate of hydrolysis at pH 7 can be calculated on the assumption that the reaction rate is proportional to the hydroxide ion concentration at all pH, and that neither the components of buffers nor water effect the opening of the ring. Since water opens the ring in methyl ethylene phosphate (Wall, unpublished), the above assumption for potassium ethylene phosphate is unlikely to be valid. Nor are adequate kinetic data available for the enzyme. On the basis of the conviction that a crude estimate is better than none, the author suggests that, if the assumptions above were valid, the enzymatic rate of ring opening (Richards & Vithayathil, 1959) for uridine-2,3-phosphate would exceed the non-enzymatic one for ethylene phosphate by a factor of 10^{11}. For the reasons already cited, this figure probably considerably exaggerates the extent of the enzymatic catalysis; the catalysis is nevertheless real and of large magnitude.

Detailed structural information

Any detailed hypothesis for the action of ribonuclease must necessarily take into account many pieces of information and many deductions (some of which may prove misleading) which have been obtained concerning the enzyme. Richards (1958; Vithayathil & Richards, 1960) has cleaved ribonuclease with a special subtilisin into two parts, which he calls the S-protein and S-peptide; the latter consists of the twenty amino-acids from the N-terminal sequence of the original enzyme. Although neither the S-protein nor the S-peptide has enzymatic activity alone, the combination is fully active, and the equilibrium constant (Richards & Vithayathil, 1959) for the dissociation of the peptide is probably less than 10^{-9} m/l. The S-peptide then has some (but not all) the properties of an artificial coenzyme; it is not chemically bonded to the protein but is held in place by the same sort of forces as those which bind DPN^+ to alcohol dehydrogenase or thiamin pyrophosphate to carboxylase.

The forces which hold the S-peptide to the S-protein have been investigated by Richards, who has chemically modified the S-peptide and then has tested it, with the S-protein, for both enzymatic activity and for the equilibrium constant of the binding. Surprisingly, he has found that acetylation of the three amino groups (N-terminal amino group and those of two lysine residues) or esterification of the carboxyl groups (the new C-terminal one formed during cleavage and those of the glutamic acid residues) does not sharply diminish either chemical activity or binding; these results suggest that the forces holding the S-peptide to the S-protein are not primarily electrostatic. On the other hand, the oxidation of the methionine residue of the S-peptide (no. 17) to a sulfone or the conversion of this residue to a sulfonium salt with iodoacetic acid sharply decreases the binding between peptide and protein (White, 1960; Anfinsen, 1960). Richards has concluded that, in the native enzyme, the $-S-CH^3$ group is held in a hydrocarbon pocket made up of side-chain residues such as the isopropyl groups of valine, etc. If the $-S-CH^3$ group

is converted to a polar one, it is then repelled rather than attracted by its lipophilic site. The importance of such non-polar binding has long been recognized, but has here been demonstrated as of large magnitude.

Conversion of the asparagine residue, no. 15, to an aspartic acid group was accompanied by complete loss of enzymatic activity (Richards, 1958; Vithayathil & Richards, 1960). This group must then be assigned an important role in the enzymatic process, and must either be part of the catalytic site or else play a specific role in the binding of the S-peptide to the S-protein, or of the substrate to the holoenzyme.

Additional information concerning the internal bonding of the chains has been supplied by the discovery (White, 1960; Anfinden, 1960) that the disulfide bonds in ribonuclease can be split by reduction, and re-formed by oxidation, with substantial reactivation of the enzyme. These facts strongly suggest that the folding of the molecule occurs naturally, and that the disulfide bonds serve to stabilize a configuration which is inherently a moderately probable one.

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Present evidence suggests that the amino acids near the carboxyl end of the sequence are also essential to enzymatic activity. Barnard & Stein (1959; Stein & Barnard, 1959; Gundlack *et al.*, 1959) have found that, under certain conditions, bromoacetate attacks ribonuclease only at the histidine residue, no. 119 in the sequence (the fifth amino acid before the C-terminal valine residue), and that this modification results in complete loss of enzymatic activity. This imidazole ring may serve as a basic catalyst to remove a proton from the 2′-hydroxyl group of a ribose residue and so promote the internal displacement which leads to the cyclic phosphate (Todd, 1956; Witzel, 1960). Further, Anfinsen (1956) has offered experimental evidence which suggests that the aspartic acid, fourth from the C-terminal end of the chain, is essential to enzymatic action. Ribonuclease is therefore probably constructed so that the C-terminal and N-terminal end are close together; the asparagine at 15, the histidine at 119 and the aspartic acid at 121 are involved in essential functions, and the methionine group at 17 is buried in a hydrocarbon pocket in the protein.

Another feature of the amino acid sequence in ribonuclease is the succession of cationic groups near the middle of the chain. The side-chains of three lysine and two arginine molecules occur in a space of eleven residues, between nos. 31 and 41. Since ribonucleic acid is a polyanion, one may hazard the guess that the polycationic section of the enzyme is part of the binding site. If this hypothesis should prove correct, then this section too will be near the other important residues.

Furthermore, the various helical sections of the enzyme should be bound together in some sensible fashion—perhaps by bringing together hydrocarbon-like residues on adjacent chains and allowing the polar residues to face outward toward the solvent. Probably the chains should not lie strictly parallel, but should cross one another at a 20° angle, to allow the various side-chains to "mesh". Finally, some consideration must be given to a reasonable chemical mechanism for the formation and hydrolytic opening of cyclic esters of phosphoric acid attached to pyrimidine nucleotides, so as to explain the enzymatic activity and specificity.

The molecular models

Scheraga (1960) has recently proposed a three-dimensional molecular model of ribonuclease. It was constructed to contain the maximum (65 per cent) of right-handed helix which can be tolerated by the requirements of the disulfide bonds present and by the necessity of making bends or corners between helical sections. The helical portions were bent so as to bring the head and tail portions of the enzyme together, and to achieve a molecule of roughly the correct dimensions. This design has now been constructed with Pauling–Corey space-filling models. As always, it is easier to criticize than to create, and the discussion below should not be allowed to detract from the value of considering a detailed structure in just the terms Scheraga employed.

The optical activity argues against the large amount of right-handed helix employed. The model constructed more or less faithfully from Scheraga's diagrams is a remarkably open structure, with large "holes"; although these holes could be filled with water, much of their surface is hydrocarbon, and will not bond well to the solvent. The methionine residue, no. 17 in Richards's N-terminal sequence, is fairly well located in a moderately hydrophobic region. But the asparagine at 15 and the histidine at 119 (which, as discussed above, must be important to enzymatic activity) are far separated, and are on the outside of the enzyme where it is unlikely that they can contribute in any significant way to chemical reaction. (These two residues are marked with arrows 1 and 2 in Fig. 1.) Furthermore, the binding site (residues 31–41, marked with arrow 3) is far separated from the catalytic regions.

An alternative model has been proposed by Parks (1960). However, the sequence of reactions and the chemical intermediates he has postulated are not those suggested by the non-enzymatic chemistry.

A complete solution of the problem may not be achieved until a detailed X-ray analysis (comparable to that for myoglobin (Bodo *et al.*, 1959)) has been obtained. But perhaps current studies of structure, of the effects of chemical modifications on enzyme activity, and of reaction rates may soon be combined with studies of the chemistry of simple phosphate esters and the construction of scale models to yield a coherent hypothesis of the action of ribonuclease.

ESTERASES AND PROTEASES

Structure

The determination of the amino-acid sequence in chymotrypsin, trypsin and other proteases and esterases is not yet far advanced (Hartley, 1959; Schaffer *et al.*, 1946; Oosterbaan & van Adrichem, 1958; Oosterbaan *et al.*, 1958; Dixon *et al.*, 1958; Jolles *et al.*, 1959; Hill *et al.*, 1959; Sorm, 1959; Mikes *et al.*, 1960; Vanecek *et al.*, 1960). The reaction of these enzymes with diisopropyl fluorophosphate (DFP) (Jansen *et al.*, 1950) and similar reagents phosphorylates a serine residue which is present in the tetrapeptide

-gly-asp-ser-gly-.

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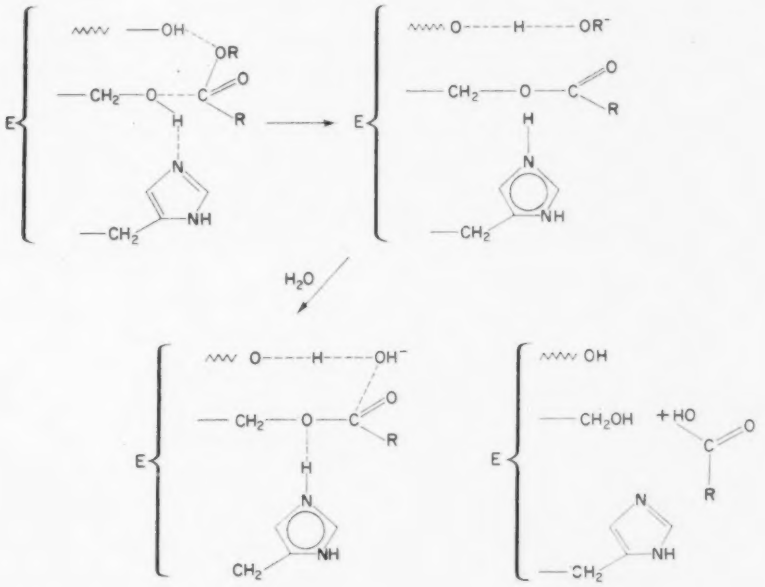
Reaction of the enzyme with *p*-nitrophenylacetate (Balls & Wood, 1956) and similar reagents acylates this same serine. Although some investigators question whether such esters are formed in the enzymatic process (Bernhard *et al.*, 1960), the concensus holds that the esterified enzyme is an obligatory intermediate in the overall reaction.

Mechanism

Special techniques for the study of fast reactions have allowed the separation of two steps in the process (Hammond & Gutfreund, 1955; Gutfreund & Sturtevant, 1956a, b; Gutfreund & Hammond, 1959): acylation of the enzyme and hydrolysis of the acylated intermediate. The pH-rate profiles (Cunningham & Brown, 1956; Schonbaum *et al.*, 1959) for both steps suggest the intervention of a histidine residue (Erlanger, 1960; Bruce & Schmir, 1959) and an informed guess as to the reaction mechanism is shown below (Bender *et al.*, 1960; Neurath & Hartley, 1959)



Details of the process

The inhibition (Neurath & Schwert, 1950) of chymotrypsin is weaker with negatively charged than with uncharged inhibitors (Foster & Niemann, 1955). Niemann therefore suggested that the enzyme may hold a negative charge in close proximity to the active site. However, the assumed anionic site has an apparent pK in the neighborhood of 7, and a normal carboxylate ion would not be discharged at this pH. Of course, in a protein molecule, the pK of an acid may easily be displaced by electrostatic or other interactions with the nearby chemical sites.

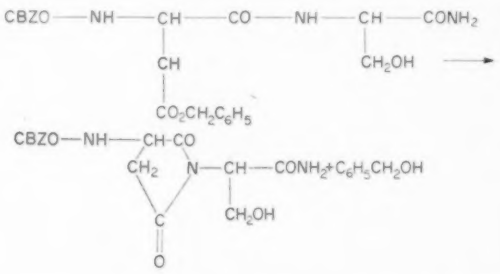
Alternatively, the apparent pK may arise if a histidine molecule is in fact required for enzymatic activity, since this residue will carry a positive charge at pH less than 7; at low pH, this positive charge will compensate electrostatically for the negative charge on the carboxylate residue.

Since an aspartic acid residue is adjacent to the reactive serine, it may supply the needed anionic center. Incidentally, this group might function to make the product-enzyme complex electrostatically unstable, and so force the product from the enzyme surface. Although such speculation is entirely unproved, it fits the limited data available.

Chemical model systems

Two types of chemical models have been extensively studied. Imidazole strongly catalyzes the hydrolysis of *p*-nitrophenyl acetate (Bender & Turnquest, 1957; Bruice & Schmir, 1957; Bender, 1960), and this catalysis is sharply enhanced when both reactants are present in the same molecule (Bruice & Sturtevant, 1958, 1959; Bender, 1957; Morawetz & Zimmering, 1954; Gaetjens & Morawetz, 1960; Swain & Brown, 1952). Mechanisms directly and indirectly related to these model systems (such as that shown above) have been extensively discussed elsewhere (Bender *et al.*, 1960; Bender, 1960).

An entirely different type of model system was developed by Katchalski, Bernhard and collaborators (1959). They discovered that the rate of hydrolysis in neutral solution for the benzyl ester of carbobenzoxyaspartylserinamide is about a million times greater than that of benzyl propionate, and they offered convincing evidence that the reaction proceeds by way of an imide as intermediate.



More detailed examination of the data reveals that the hydroxyl group and the C-terminal amide groups of serinamide are important to the catalysis. In a somewhat similar study, Anderson *et al.* (1960) have found that O, N-diacetyl serinamide is hydrolyzed about 1000 times as rapidly as are simple esters, and that the hydrolysis in neutral solution is catalyzed by imidazole. A possible role for the serine hydroxyl group and for the amide group in the hydrolysis of the benzyl ester of carbobenzoxyaspartylserinamide might require these groups to hydrogen-bond the incipient negative charges which arise during the formation of the imide, and so stabilize the transition state. Such stabilization has been postulated for other systems (Kupchan & Johnson, 1956; Henbest & Lovell, 1957).

The best model systems take advantage of the high rate of intra- as contrasted to intermolecular reactions. If a substrate of molecular weight 100 is present at a concentration of 10^{-5} M, the loss of translational entropy in the formation of the transition state in a bimolecular enzymatic reaction exceeds 55 e.u., and this loss is only partially compensated by the entropy of the new internal rotations and vibrations in the enzyme-substrate complex, or by the requirements of forming cyclic intermediates. Some caution need be exercised, however, in examining the thermodynamic data now available for the formation of enzyme-substrate complexes. Frequently in solution the situation is complicated by electrostatic interactions which affect both entropy and energy. The combination of two ions of opposite charge, for example, is accompanied both by a decrease in translational entropy and by an increase in electrostatic entropy, since water molecules which were "frozen" about the individual ions are set free in the uncharged and therefore relatively less polar transition state. Since the driving force for the formation of an enzyme-substrate complex may well be electrostatic, the experimentally determined entropy of formation of an enzyme-substrate complex may prove to be the sum of several partially compensating terms (Hammett, 1940).

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Additional rate factors

Bruice (1960) has recently suggested that the rate of hydrolysis of esters and amides might be increased by altering their conformation. Lactones hydrolyze much more rapidly than do esters; the rate for δ-valerolactone is 6500 times that of ethyl propionate (Huisgen & Ott, 1959). Normal esters hold the alkyl group between the carbonyl oxygen atoms (Huisgen & Ott, 1959; Curl, 1959); if an enzyme can twist its substrate into a conformation analogous to that of a lactone, the rate of hydrolysis may easily be increased 100-fold. Furthermore, the hydrolysis of lactones, in contrast to that of esters, is accelerated by bases (Curl, 1959) such as imidazole; the same might be true of esters with a twisted conformation.

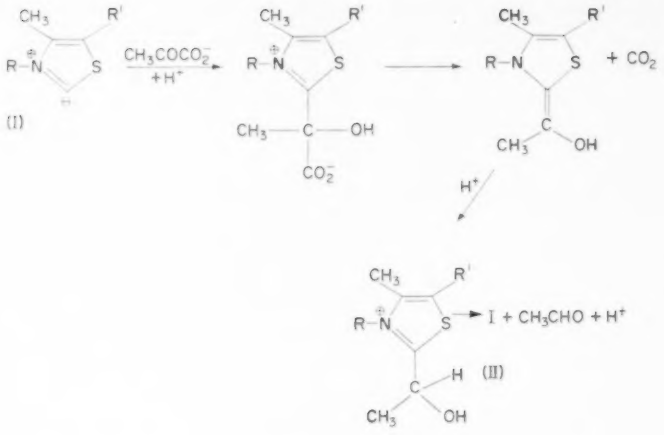
CARBOXYLASE

Chemical model systems

Considerable progress has been made in understanding the mechanism of action of carboxylase. The role of thiamin pyrophosphate in this and similar enzymes was advanced by Breslow (1958), and the subject recently thoroughly reviewed by Metzler (1960). In brief, Breslow has postulated that thiamin pyrophosphate ionizes at the 2-position of the ring of the thiazolium ion, and that the yield I condenses with pyruvate to form an intermediate which decarboxylates and yields a 2-hydroxyethyl thiazolium salt, II, as an intermediate in the production of acetaldehyde.

In the structure overleaf, R represents the pyrimidine portion of the coenzyme, and

$$R' = -CH_2-CH_2-O-PO_2-O-PO_3\equiv$$



Enzymatic studies

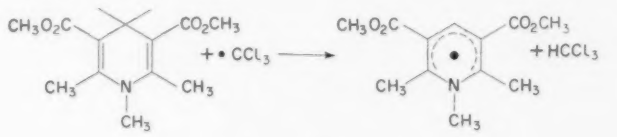
Two new pieces of evidence support this mechanism. Carlson & Brown (1960) (Krampitz *et al.*, 1958; Breslow & McNelis, 1959) have shown that the hydroxyethyl thiazolium salt, II, is present in extracts of *Escherichia coli* to the extent of about 60–75 per cent of the total thiamin present. Fry (1960) has studied the decarboxylation of pyruvate by carboxylase in D_2O. He found that a highly purified (but still not pure) enzyme from brewer's yeast catalyzes the exchange of hydrogen for deuterium in the 2-position of the thiazolium ring system. In these experiments, Fry allowed the enzyme to come into contact with D_2O for only 30 sec at pH 4·5. The thiazole was cleaved from the coenzyme under acid conditions, where control experiments showed that little non-enzymatic exchange at the 2-position of the ring occurs. On analysis, the thiamin showed approximately one atom of deuterium per molecule. These experiments demonstrate that the enzyme catalyzes the preliminary ionization required by Breslow's theory. Here the studies of model systems (Breslow, 1958; Ukai *et al.*, 1943; Mizuhara *et al.*, 1951; Breslow & McNelis, 1960; White & Ingraham, 1960) first revealed an enzymatic mechanism which has now (Carlson & Brown, 1960; Krampitz *et al.*, 1958; Breslow & McNelis, 1959; Fry, 1960; De Tar & Westheimer, 1959) been amply supported.

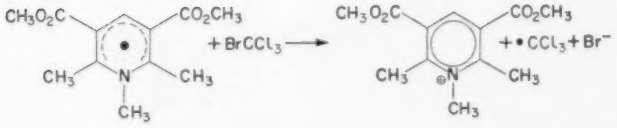
DEHYDROGENASES

Chemical model systems—Diphosphopyridine Nucleotide

The mechanisms of reaction of enzymes requiring DPN^+ and TPN^+ have been shown to proceed stereospecifically with direct hydrogen transfer from substrate to coenzyme (Vennesland & Westheimer, 1954; Kaplan, 1960). However, little progress has subsequently been made in establishing the course of the reactions: in particular, the question of whether a particular enzymatic process occurs by a free-radical or a polar mechanism is still debatable. Thiobenzophenone (Abeles *et al.*, 1957) and malachite green (Mauzerall & Westheimer, 1955)

had been reduced by dihydropyridines in good yield with direct hydrogen transfer, and pyruvic acid (Mauzerall & Westheimer, 1955) and benzoyl formic acid (Abeles & Westheimer, 1958) have similarly been reduced by a 1,4-dihydropyridine derivative, but in poor yield. The reduction of pyruvic acid by a special N-alkyl-1,4-dihydropyridine (but also in poor yield) has been achieved by Wallenfels & Hofmann (1959) and other model reactions have been reported (Spiegel & Drysdale, 1960; Wallenfels & Gellrich, 1959; Frisell & Mackenzie, 1959; Berson & Brown, 1955). More recently, the photochemical reduction of carbon tetrachloride and of bromotrichloromethane by dihydropyridine derivatives such as 1,2,6-trimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine has been studied (Westheimer, 1959; Kurz *et al.*). In purified methanol, the quantum yield varied with experimental conditions from about 10 to 100. Furthermore, the reaction is inhibited by dihydroanthracene, a known inhibitor for reactions of the trichloromethyl radicals. The reaction is presumably a free-radical chain process.





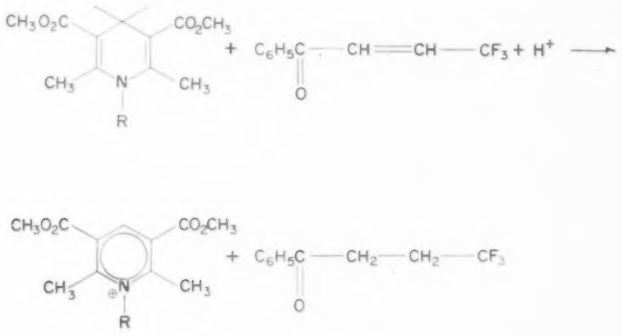
The finding supports the conclusion of Schellenberg & Hellerman (1958) and others (Commoner *et al.*, 1957) that reactions of 1,4-dihydropyridines may occur by a free-radical process. Whether, or in what instances, such a free-radical intermediate is involved in enzymatic processes remains unknown.

An interesting series of reactions has been carried out by Braude, Hannah & Linstead (1960) and others who studied reductions with derivatives of dihydropyridine, and especially with the "Hantzsch compound", 2,6-dimethyl-3,5-dicarboethoxy-1,4-dihydropyridine. This compound is smoothly and rapidly oxidized to the corresponding pyridine by maleic anhydride (and similar compounds) with concomitant reduction of the double bond of the olefin. The reaction occurs under mild conditions with maleic anhydride, maleic acid, ethyl maleate, fumaric acid, and other activated olefins. Braude *et al.* expressed the opinion that these reactions are ionic in character, and correspond to the transfer of a hydride ion from the dihydropyridine to the olefin.

A similar oxidation–reduction process had been studied for some years in the laboratories at Harvard. Graves (unpublished) found that the Hantzsch compound reduces benzoylacrylic to benzoylpropionic acid. When the reaction was carried out

with 2,6-dimethyl-3,5-dicarbomethoxy-4,4-dideutero-1,4-dihydropyridine, a deuterium atom was transferred to the resulting benzoylpropionic acid (Klinedinst, unpublished). Since this deuterium could not be removed under mild conditions with alkali, the reduction presumably places the deuterium atom beta to the ketonic carbonyl group. This reduction therefore closely parallels the enzymatic reduction of testosterone. In the latter reaction, McGuire & Tomkins (1960) have shown that in tritiated water the process, catalyzed by TPNH and by a partially purified enzyme from rat liver, leads to the incorporation of one and only one atom of tritium into the steroidal hormone; furthermore, this tritium atom is easily removed by alkali under mild conditions. Since only one tritium atom was incorporated from the solvent, the other hydrogen atom presumably was supplied by the TPNH in a position beta to the ketonic group. The reduction of benzoylacrylic acid is thus a possible model for the enzymatic process.

Although compounds such as benzoylacrylic acid, dibenzoyl methane, and maleic anhydride are reduced, simple α, β-unsaturated ketones, such as benzalacetophenone, are resistant to the action of the Hantzsch compound. In an attempt to determine the role of the carboxyl group in benzoylacrylic acid, Norcross (unpublished) and Klinedinst (unpublished) prepared 1-benzoyl-3,3,3-trifluoropropene-1. This compound readily undergoes reduction by the Hantzsch compound; these facts suggest that the effect of the carboxyl group in benzoylacrylic acid is electrostatic in nature.



A step nearer to a true model system has been achieved by the reduction in good yield of benzoyltrifluoropropene by 1,2,6-trimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine (Norcross, unpublished) in methanol solution at 63°. (Equation above, with R = CH_3.) Although the mechanism for these processes has not been proved, the strong influence of the electronegative trifluoromethyl group at least suggests the transfer of a hydride ion from the dihydropyridine derivative to the olefinic double bond. However, neither for carboxylase nor for enzymes which require DPNH is the structure of the enzyme known, and the function of the protein in promoting the reactions between coenzyme and substrate remains to be discovered.

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STEROID HORMONE BIOSYNTHESIS

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STEROID hormones are formed in particularly specialized glands in a well-regulated, usually balanced system, and in a well-ordered sequence. The individual biosynthetic pathways for the formation of progesterone, corticoids, androgens and estrogens are intimately interrelated, and the biosynthetic capacities of the active tissues show a good deal of overlapping.

It is possible to define a central, almost obligatory, plan for the formation of all steroidal hormones to which specialized specific pathways are added. This common pathway concerns the formation successively of cholesterol and pregnenolone, and in this area newer findings indicate that two hydroxylation reactions occur, followed by the splitting of the C-20, C-22 carbon–carbon bond to form pregnenolone and isocaproic acid.

Solomon *et al.* (1956), in a preliminary communication, had indicated by dilution experiments that 20α-hydroxycholesterol (previously designated as 20β-hydroxycholesterol) was an intermediate between cholesterol and pregnenolone. These experiments were done by incubating cholesterol-^{14}C with bovine adrenal homogenates and diluting with 20α-hydroxycholesterol, 22α-hydroxycholesterol, 22β-hydroxycholesterol, 22-ketocholesterol, 24β-hydroxycholesterol, and 24-ketocholesterol. After isolating and purifying the various added compounds, only 20α-hydroxycholesterol could be shown to contain radioactivity. We have now established that 20α-hydroxycholesterol-22-^{14}C-7-^{3}H can be converted to pregnenolone-^{3}H and isocaproic acid-22-^{14}C by bovine adrenal supernatant (Shimuzu *et al.*, 1960; Shimuzu *et al.*, 1960). These studies have indicated, also, that a cholesterol intermediate containing oxygen at C-20 and C-22 yielded pregnenolone and isocaproic acid by incubating this substrate with a bovine adrenal homogenate. Since Solomon *et al.* (1956) were unable to demonstrate the presence of ^{14}C-labeled 22α- or 22β-hydroxycholesterol or 22-ketocholesterol, we have tentatively concluded that 20α-hydroxylation may precede 22-hydroxylation.

On the basis of the still fragmentary evidence, the pathway may be described as cholesterol → 20α-hydroxycholesterol → 20, 22(?)-dihydroxycholesterol (→ 20α-hydroxy, 22-ketocholesterol) → pregnenolene + isocaproic acid. This pathway appears to be general for the adrenal, testis, ovary and placenta. Since it is indicated that ACTH, in part, regulates the conversion of cholesterol to pregnenolone in the adrenal, it may be expected that the same steps are regulated by gonadotropin in the testis, ovary and perhaps even in the placenta. The proof is yet to be found for these points.

CORTICOID PRODUCTION

The formation of corticoids has been elucidated to a reasonable extent, but certain questions still remain unanswered. To deal with the more clearly established facts, it is known that pregnenolone is oxidized with the aid of 3β-ol-dehydrogenase, and that the progesterone so formed undergoes a series of hydroxylation reactions. These can be best represented by Fig. 1.

It is generally believed that the 17α-hydroxylation in the adrenal is a property of the fasciculata and reticularis and that the glomerulosa zone is lacking in this enzyme. 18-Hydroxylation appears to be specific for the glomerulosa, the site of aldosterone biosynthesis. It is possible that the failure of the inner zones to produce aldosterone may be due to the lack of 18-hydroxylase or perhaps the absence of the proper 18-dehydrogenase necessary to oxidize the 18-hydroxy group to the corresponding aldehyde.

The inability of the 17α-hydroxylase to act on 21-hydroxylated substrates is well established. Though earlier papers had reported this reaction, for example, the conversion of corticosterone to cortisol, this has been demonstrated to be in error (Eichhorn & Hechter, 1957). The reason for the lack of 17α-hydroxylation of a 21-hydroxylated substrate is not apparent and may well require further insight into the mechanisms involving hydroxylation of positions adjacent to oxygen functions.

The control of corticosterone and cortisol formation by adrenal tissue may be exercised in part by the products of the biosynthetic reactions. It can be demonstrated, at least *in vivo*, that the ACTH stimulation of the rat and human gland is inhibited by administration of high levels of corticoids (Peron *et al.*, 1960).

In the biosynthetic sequence leading to corticoids and other steroid hormones, hydroxylation reactions are of prime importance. Certain conclusive studies have been published which have established the following points: the oxygen for the reaction is derived from molecular oxygen (Hayano *et al.*, 1955; Hayano, Saba *et al.*, 1956; Hayano, Saito *et al.*, 1956; Sweat *et al.*, 1956; Hayano *et al.*, 1958; Hayano & Dorfman, 1954; Slaunwhite & Samuels, 1956) and the stereospecific introduction of oxygen occurs as a direct substitution of the hydrogen by an hydroxyl group without inversion (Hayano & Dorfman, 1954).

One outstanding problem of hydroxylation involves substrate specificity. To pose the problem more precisely with respect to 11β-hydroxylation, do there exist separate enzymes for the 11β-hydroxylation of deoxycorticosterone, 11-deoxycortisol, and Δ^4-androstene-3,17-dione, or is a single enzyme involved? This problem is of theoretical as well as practical significance. As an example, one may consider variants of congenital adrenal hyperplasia in which 11β-hydroxylase(s) are low. If one and only one enzyme exists for all three substrates, then the decrease will be reflected in all three products, whereas if all the enzymes were different, various combinations would be expected. Cortisol could be low while corticosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione production could be normal or even increased.

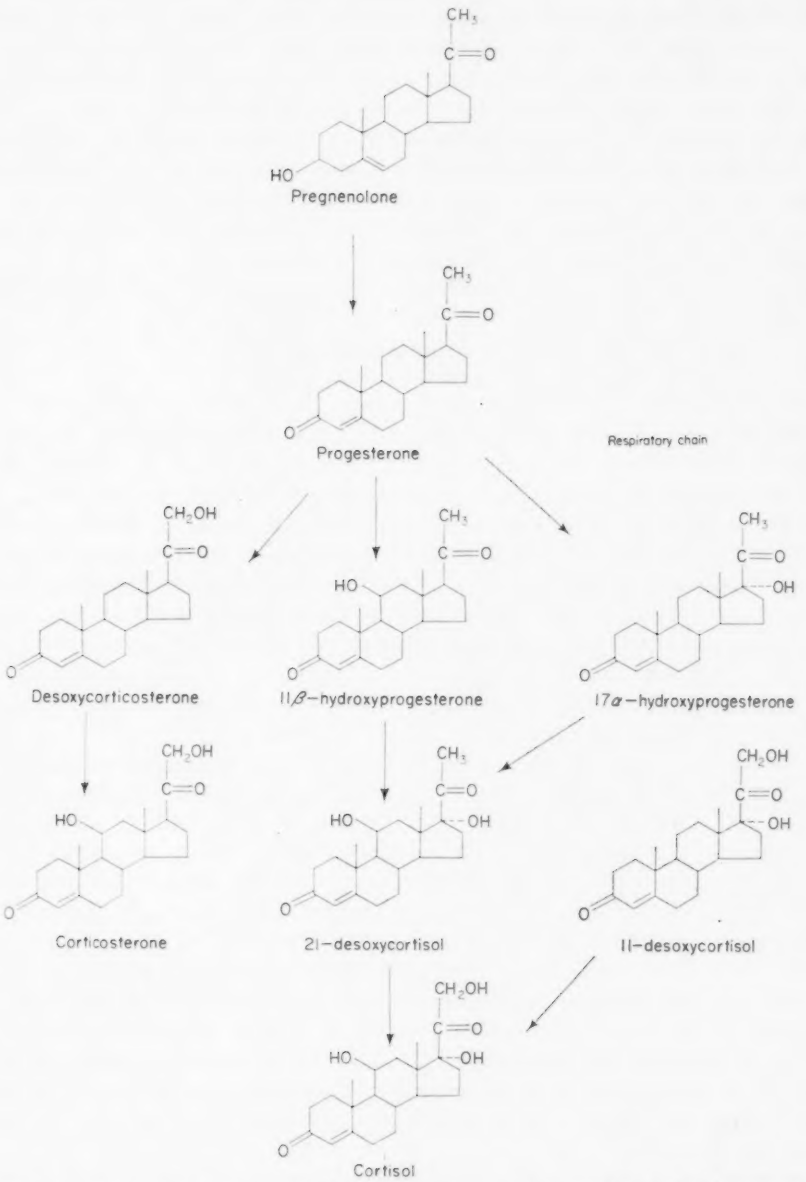

FIG. 1. Biosynthesis of cortisol and corticosterone. Biosynthesis of aldosterone (not shown) involves 18-hydroxylation (see text).

ANDROGEN BIOSYNTHESIS

Considerable progress in androgen biosynthesis has been made in the past few years, particularly as a result of the detailed study of abnormal tissues. Five distinct mechanisms have been established or indicated. The mechanism which has been described for all steroid-producing tissues, and first reported by Slaunwhite & Samuels (1956) and soon thereafter by Savard *et al.* (1956), involves the 17α-hydroxylation of progesterone, the formation of Δ^4-androstene-3,17-dione by a desmolase reaction, and the reduction of the 17-keto group to testosterone. This pathway in testis (Slaunwhite & Samuels, 1956; Savard *et al.*, 1956) and in ovaries (Solomon *et al.*, 1956; Kase *et al.*, in press) is established.

Evidence for the second pathway involving the formation of dehydroepiandrosterone rests on secure grounds. Incubation of a homogenate prepared from a human adrenal adenoma with pregnenolone-7-^{3}H resulted in the formation of dehydroepiandrosterone-^{3}H (Goldstein *et al.*, 1960). The same conversion was reported by Burstein & Dorfman (1960), who injected the ^{3}H-labeled substrate into a virilized woman bearing an adrenal adenoma and isolated tritiated dehydroepiandrosterone from her urine in good yield. The step 17α-hydroxypregnenolone to dehydroepiandrosterone has been established by Solomon *et al.* (1960). The isolation of dehydroepiandrosterone from testis by Neher & Wettstein (1960) seems to cast doubt on the thesis that this androgen is exclusively of adrenal origin. However, one interpretation is that the androgen as the sulfate of adrenal origin is hydrolyzed to the free steroid by the testicular sulfatase, and that it would be possible to isolate the minute quantity of 7·5 μg/kg of testis, although the dehydroepiandrosterone is not produced in the testis. Direct studies showing the conversion of pregnenolone to dehydroepiandrosterone have not been done and results of such experiments would be of interest.

The third pathway involving the conversion of progesterone to testosterone acetate and finally to testosterone is indicated from the studies of Kase *et al.* (1960) who found that the incubation of progesterone-7-^{3}H and 17α-hydroxyprogesterone-4-^{14}C, with a homogenate prepared from human polycystic ovaries, yielded testosterone containing a higher ratio of ^{3}H/^{14}C than that found for the Δ^4-androstene-3,17-dione simultaneously formed. This strongly indicates that testosterone could be formed without the prior formation of Δ^4-androstene-3,17-dione. Such a mechanism has now been established by Fonken *et al.* (1960), who isolated the necessary testosterone acetate intermediate in the transformation of progesterone to testosterone by *Cladosporium resinae*. If this mechanism does pertain to androgen biosynthesis, then it may be expected that testolactone is a naturally occurring compound, since the same reaction on Δ^4-androstene-3,17-dione would form the lactone. Experiments to test this hypothesis are yet to be done.

A possible fourth mechanism is the direct formation of dehydroepiandrosterone from cholesterol without the necessity of a C_{21} intermediate. It is visualized that 17α, 20α-dihydroxycholesterol may be the intermediate. This pathway still remains an interesting hypothesis.

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The fifth and final pathway is related to peripheral formation of androgens from nonandrogenic compounds by enzymes in such tissues as liver and muscle. Oertel & Eik-Nes (1959) have suggested that DHA may be formed from pregnenolone and 17α-hydroxypregnenolone by striated muscle tissue. This has not yet been repeated, and in one experiment aimed at confirming this observation, excellent yields of the 50 reduced derivatives of 17α-hydroxypregnenolone were isolated after incubation of the 17α-hydroxy substrate with rabbit muscle strips, but dehydroepiandrosterone could not be detected (Thomas *et al.*, 1960).

The formation of C_{19} from C_{21} steroids with liver preparations is well documented. C_{21}-compounds having oxygen functions at C-17 and C-20 are metabolized to 17-ketosteroids (Dorfman, 1957). Many *in vivo* experiments have been reported using the following substrates: cortisone, cortisol, 21-desoxycortisone, 21-desoxycortisol, 11-desoxycortisol and 17α-hydroxyprogesterone. The yield of 17-ketosteroids from C_{21} steroids possessing the Δ^5-3β-hydroxyl groups is severely reduced. *In vitro* methods have been quite illuminating, such as those of Caspi & Hechter (1954, 1956), Forchielli & Dorfman (1956) and Forchielli *et al.* (1955), where 11-desoxycortisol incubated with liver homogenates has yielded androsterone, androstene-3,17-dione, Δ^4-androstene-3,17-dione and etiocholanolone. The cellular location of the desmolase has not been established.

The recent findings in the biosynthesis of androgens has clarified some aspects of normal and abnormal physiology. These advances have permitted the formulation of a more general hypothesis of virilism in girls and women, puberty in males, and the change in virility in men in advancing years. It can now be stated that *the androgen* in humans is *testosterone*, and that other androgens such as dehydroepiandrosterone, Δ^4-androstene-3,17-dione, and 11β-hydroxy-Δ^4-androstene-3,17-dione are relatively inactive in man. Testosterone biosynthesis can take place in the testis, ovary and adrenal. The testosterone of the adult testis is probably of the order of 3·5 mg per day, while that of the adrenal is somewhere in the order of 0·5 mg, so that in a normal adult male the total production is probably 4·0 mg. In the adult normal woman it is likely that the ovaries plus the adrenals produce about 1 mg of testosterone per day, with each gland contributing an equal quantity. Perhaps as little as an additional production of 1 mg of testosterone per day in a woman produces hirsutism, while a total of 3–4 mg total production per day produces frank virilism, the intensiveness being in part conditioned by the time over which this excessive concentration is active. Of particular importance is the fact that although a few milligrams of testosterone per day may produce bizarre effects in woman, conventional urinary or blood analyses cannot indicate the true effective androgen concentration in the tissue fluids. This is so because conventional methods determine dehydroepiandrosterone, a weak androgen, or androsterone and etiocholanolone, which are catabolites of three different tissue androgens, namely dehydroepiandrosterone, Δ^4-androstene-3,17-dione, and testosterone, of which the latter is lowest in amount. Actually in a normal man only 2 mg of the 12 mg of 17-ketosteroids in a 24-hr sample of urine are due to endogenously produced testosterone. In a normal woman, of 10 mg of urinary

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17-ketosteroids per day, only 0·5 mg is due to the testosterone produced. In view of this, it is not surprising that many hirsute women have normal titers of 17-ketosteroids. This is also true of women with frank virilism in conditions such as arrhenoblastoma (Savard *et al.*, 1960), polycystic ovaries (Kase *et al.*, unpublished; Castro *et al.*, unpublished) and various types of adrenal hyperactivity, including those involving tumors.

Similar situations have been observed in cases of precocious puberty in boys, that is, frank advanced sexual development with no significant increases in urinary 17-ketosteroids.

In another part of this paper, evidence was presented for a pathway of androgen biosynthesis involving a direct transformation of progesterone to testosterone without a C_{21}-17α-hydroxy intermediate. This mechanism may well be considered to be *the* adult mechanism for testosterone formation and explain some important new data on androgen production by immature and mature testes. Lindner (1939) has demonstrated that the calf and bull testis on the basis of μg of Δ^4-androstene-3,17-dione plus testosterone per mg of tissue produce equal amounts of hormones. The real difference, however, between the immature and mature testis is the ratio of the highly active testosterone to the relatively inactive Δ^4-androstene-3,17-dione. For the calf testis this ratio is 0·7, while for the bull testis the ratio is greater than 15. When the respective testes were stimulated with gonadotropin, the total C_{19} production, as expected, was increased in both and the ratio was preserved. These data may be interpreted to mean that different pathways are operative in the immature and adult testis, and that in the former tissue the major pathway involves the sequence progesterone $\rightarrow$ 17α-hydroxyprogesterone $\rightarrow$ Δ^4-androstene-3,17-dione $\rightleftarrows$ testosterone, which may be termed the immature or primitive pathway, while the major pathway in the latter gland is progesterone $\rightarrow$ testosterone acetate $\rightarrow$ testosterone $\rightleftarrows$ Δ^4-androstene-3,17-dione, which may be called the adult male pathway. This hypothesis would explain the ratio differences between the two groups of testes and further explain why stimulation with gonadotropin increases androgen production without changing the ratio. This hypothesis further points to a testicular development during puberty which involves an "induction" of the adult mechanism. What the factors may be which increase the effective concentration of the adult mechanism, is, of course, not known, but the possibility that this "induction" may be due to a pituitary factor should be considered analogous to the ACTH–adrenal relation described by Kass *et al.* (1954).

Further consideration of the adult mechanism in relation to certain abnormal states of the ovary and adrenal seems in order. The polycystic ovary, arrhenoblastoma, and certain hyperactive adrenals may produce their excessive androgenic activity by this adult pathway, which means that these abnormal tissues behave in a sense like a testis.

Some years ago Hill (1937) and Hill & Strong (1940) demonstrated that the mouse ovary, which produces only traces of androgens *in situ*, can be made to produce excessive quantities of androgens by transplanting the gland to the ear. This change in site stimulated the gland to produce sufficient androgens to

maintain the prostate and seminal vesicles of the castrated male mouse. The reason for this transformation is not known, but the lowered temperature is a factor. It would be of interest to know whether the temperature change produces an increase in the enzymes related to the direct conversion of progesterone to testosterone.

C_{19}-Δ^{16} STEROIDS

This special group of steroids has been a complete enigma from the point of view of biosynthesis until recent developments in our laboratory which have proved that these steroids are derived from cholesterol and pregnenolone. Prelog & Ruzicka (1944) isolated two compounds of this series, the Δ^{16}-androsten-3α-ol and the Δ^{16}-androsten-3β-ol from boar testis. The Δ^{16}-androsten-3α-ol has been isolated from human urine on a number of occasions (Miller *et al.*, 1953; Brooksbank & Haslewood, 1949; Mason & Schneider, 1950), and the important facts that were indicated from these reports were that this Δ^{16}-steroid was present in higher concentrations in men's urine than in women's urine, but that the highest amounts were present in virilized women. In such a masculinized woman, bearing a 700 g adrenal adenoma, who excreted about 1 g of 17-ketosteroids per day, some 20 mg of Δ^{16}-androsten-3α-ol were isolated from a single day's urine. When cholesterol-4-^{14}C and pregnenolone-7-^{3}H were administered intravenously the isolated Δ^{16}-androsten-3α-ol contained both the ^{14}C and ^{3}H labels (Burstein & Dorfman, 1960). Mevalonic acid-5-^{14}C administered by the same route yielded the same Δ^{16}-steroid labeled with ^{14}C (Burstein & Dorfman, unpublished). These experiments proved that the biosynthetic origin of the Δ^{16}-steroid was similar to that of other steroid hormones and was consistent with a rather intimate metabolic relation to testosterone. The working hypothesis then was that testosterone was in fact the precursor of these unique steroids. Two studies have now been completed that demonstrate for the first time that a steroid dehydration reaction is possible and that testosterone is a precursor. Testosterone-4-^{14}C incubated with human liver homogenate yielded $\Delta^{4,16}$-androstadien-3-one (Stylianou *et al.*, unpublished) and the same labeled substrate incubated with a rat testis homogenate permitted the demonstration of $\Delta^{4,16}$-androstadien-3-one and Δ^{16}-androsten-3β-ol. These studies establish the dehydration reaction in ring D, but the mechanism is still obscure. Of interest is the fact that the Δ^{16}-steroids were produced in the testis, a finding which is in accord with the first isolation from this gland by Prelog & Ruzicka (1944). Still to be evaluated is the proportion of Δ^{16} compound coming from the steroid-producing gland and that from peripheral metabolism. At any rate, the Δ^{16}-steroids, in part, represent endogenously formed, plus circulating, testosterone and thus could give a truer and better measure of effective androgen in the body fluids than conventional analyses for 17-ketosteroids.

Brooksbank & Haslewood (1960) have suggested a method for the quantitative determination of Δ^{16}-androsten-3α-ol in urine based on the reaction of Miescher (1946). This method consists of dissolving the compound or a suitable extract prepared from urine in acetic acid containing 0·5 g/100 ml (w/v) resorcylaldehyde

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and heating at 100°C with an equal volume of 5 (v/v) sulfuric acid in acetic acid. The blue color formed is measured at 585 mμ. The authors indicate that 2–3 μg of Δ^{16}-androsten-3α-ol in 4 ml of solution may be determined.

The tentative results bear out the idea that men excrete a higher urinary concentration of this steroid than women, but unfortunately some familial irregularities were observed which tend, at first look, to minimize the value of the assay as a means to evaluate the testosterone level of the body fluids.

DETERMINATION OF BLOOD TESTOSTERONE

To ascertain the effective androgen concentrations of body fluid, for reasons already documented, the direct determination of testosterone is required. This now has been accomplished by the paper chromatographic isolation of testosterone and the conversion of this steroid to estradiol-17β by a human placental enzyme preparation. The estradiol-17β thus formed, after paper chromatographic purification, is quantitatively determined by a phosphoric acid fluorescence reaction (Finkelstein *et al.*, 1961). By this means it has been possible to demonstrate that men's plasma contains 0·2 μg/100 ml, women's plasma contains 0·05 μg/100 ml, and virilized women may have plasma values 5–10 times that of normal men.

BIOSYNTHESIS OF ESTROGEN

Estrogen formation from androgens had been suspected for over 20 years and the early suggestive experiments involving *in vivo* administration of testosterone and Δ^4-androstene-3,17-dione resulted in increased excretion of urinary estrogens (Dorfman & Shipley, 1956). With the advent of labeled substrates it became possible to establish a direct relationship. This was accomplished in various experimental designs as follows: incubation of human ovarian slices with testosterone-3-^{14}C and isolation of estrone-^{14}C (Baggett *et al.*, 1955, 1956); *in vivo* administration of testosterone-3-^{14}C to a pregnant mare and the isolation of estrone-^{14}C from the urine (Heard *et al.*, 1955); *in vivo* administration of testosterone to an adrenalectomized–ovari-ectomized woman and isolation of estrone-^{14}C from the urine (West *et al.*, 1956). These experiments proved the biosynthetic relationship between testosterone and estrogens in the ovary and indicated that the reaction can take place in peripheral tissues other than endocrine glands.

Important advances were announced by Meyer (1955), who incubated 19-hydroxy-Δ^4-androstene-3,17-dione, Δ^4-androstene-3,17-dione, $\Delta^{1,4}$-androstadiene-3,17-dione, and 19-nor-Δ^4-androstene-3,17-dione with placental homogenates and ovarian follicular fluid and showed that estrone was formed. This study implied that a necessary step in the formation of estrogens was the hydroxylation of the androgen at C-19. Meyer's study has been confirmed by various investigators (Ryan, 1959) and extended by Longchampt *et al.* (1960), who incubated Δ^4-androstene-3,17-dione-4-^{14}C with a human placental preparation and isolated, in addition to estrone-^{14}C, 19-hydroxy-Δ^4-androstene-3,17-dione-^{14}C. 19-Oxo-Δ^4-androstene-3,17-dione is transformed to estrogens as readily as the

19-hydroxy derivative (Hayano *et al.*, 1960). On the basis of these observations a sequence for estrogen biosynthesis may be written as follows: Δ^4-androstene-3,17-dione → 19-hydroxy-Δ^4-androstene-3,17-dione → 19-oxo-Δ^4-androstene-3,17-dione → estrone. The steps involved in the aromatization are still unclear. It is recognized that, since the initial substrate may be testosterone, the final product could be estradiol-17β, and in fact, with the proper dehydrogenase present, both the 17-keto and 17β-hydroxy forms could be present at each step.

The mechanism described above is not the only possible route to estrogen synthesis, since 19-nor-Δ^4-androstene-3,17-dione and $\Delta^{1,4}$-androstadiene-3,17-dione may also undergo aromatization, although the yield with placental preparations is severely reduced. 19-Nor-Δ^4-androstene-3,17-dione has been detected in the follicular fluid of the mare's ovary which lends support to the idea of a biosynthetic pathway by way of this compound and, in part, different from the pathway previously described. 19-Oxo-Δ^4-androstene-3,17-dione is likely to be common to both pathways, and in this alternative mechanism this steroid probably is oxidized to the 19-carboxylic acid, and carbon dioxide split off to form the 19-nor-steroid. The aromatization of 19-nor-Δ^4-androstene-3,17-dione-4-^{14}C by human placental preparation has been accomplished (Gual & Dorfman, unpublished). Model experiments, using micro-organisms, have not yielded consistent results. Talalay (1957) has interpreted his results to indicate that 19-nor-Δ^4-androstene-3,17-dione is aromatized by direct removal of two hydrogen atoms. Similar results were found by Gual *et al.* (unpublished), who incubated 2α-hydroxy-testosterone with *B. sphaericus* and isolated 2-hydroxy-$\Delta^{1,4}$-androstadiene-3,17-dione. This would indicate that the 2β and 1α hydrogen atoms were eliminated. Kushinsky (1958), however, reported evidence for a hydroxylated 19-nortestosterone intermediate in the formation of estrone from 19-nortestosterone incubated with *Corynebacterium simplex*.

Whether $\Delta^{1,4}$-androstadiene-3,17-dione is a naturally occurring steroid is not known, but that it can be aromatized to estrone by a placental enzyme has been established with reasonable certainty (Meyer, 1955; Ryan, 1959; Hayano *et al.*, 1960; Gual & Dorfman, unpublished). It is anticipated that the mechanism for this transformation, assuming that the $\Delta^{1,4}$ compound is normally formed, involves 19-hydroxylation followed by spontaneous nonenzymatic aromatization (Longchampt *et al.*, in press).

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THE FUNCTIONAL BIOCHEMISTRY OF CELL STRUCTURES: THE CYTOPLASM

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CYTOPLASMIC STRUCTURE

THE various cells of a multicellular organism differ from one another, and differ also from unicellular organisms, so that it is not possible to speak of the cytoplasmic structure of a typical cell. Nevertheless, some subcellular structures are common to nearly all cells and must therefore have general significance; these structures, the nucleus or nuclear apparatus, the oxidative bodies (mitochondria) and the small granules (ribosomes), are the particular concern of this review; all three organelles play a significant part in protein and nucleic acid biosynthesis.

Were it possible to find a cell with no organized structure other than nucleus, mitochondria and ribosomes, this cell would have obvious attraction for biochemists but unfortunately such cells do not exist. Tumour cells such as the Ehrlich, Krebs or HeLa cells are, however, less complex than fully differentiated cells such as the parenchymal liver cell and they have the added advantage that they may be readily obtained as uniform cell populations. At the National Institute particular attention has been paid to the Krebs II tumour cell in the ascites form. This cell type may be readily propagated in the peritoneal cavity of the mouse (Klein & Klein, 1951) and can be maintained for several days as suspensions in glucose-buffer medium (Hoskins & Sanders, 1957). Under these conditions there is neither increase nor decrease in cell numbers, but the cells are healthy as judged by their capacity to resume cell division when re-implanted in the mouse peritoneum. Eagle *et al.* (1959) have shown that tumour cells maintained in a simple medium show a sustained and uniform protein turnover of about 1 per cent/hr. Such cell preparations in which there is no growth and no cell division are probably as simple as one is likely to find for the study of protein biosynthesis.

The Krebs II cell is remarkably robust and difficult to disrupt but we have recently devised methods for either osmotic or mechanical disruption which give a high yield of apparently undamaged nuclei and a remarkably sharp separation into the four fractions shown in Table 1.

A clear separation of this type in which all the DNA is confined to the nuclear fraction and all the succinoxidase to the mitochondrial fraction is difficult to achieve with most cells and is a good test of the efficacy of the disruption and separation methods employed. The low recovery of protein is due to the presence in these cells of a glycoprotein fraction, insoluble in alkali and therefore not extracted by the methods which we have used. The analytical figures presented in Table 1 show, in common with all similar homogenate analyses, a high concentration of

RNA in the microsome fraction. However, when the Krebs cell is incubated in the presence of a radioactive precursor of RNA (^{14}C-orotic acid), very little radioactivity is found in the microsomes (Table 2). In contrast, incubation with ^{14}C-valine results in rapid labelling of protein in this fraction.

TABLE 1—ANALYTICAL COMPOSITION OF SUBCELLULAR FRACTIONS FROM KREBS II ASCITES TUMOUR CELLS AFTER DISRUPTION BY OSMOTIC SHOCK

Fraction	DNA	RNA	Protein	Succinoxidase
Nuclei (600 g)	100	12	9	0·7
Mitochondria (1000–10,000 g)	0	16	14	102
Microsomes (10,000–100,000 g)	0	61	16	0
Cell sap	0	12	34	0
Percentage recovery	100	102	74	103

Results are expressed as percentages of value for whole homogenate (data from Martin *et al.*, 1961).

TABLE 2—THE DISTRIBUTION OF TOTAL INCORPORATION AND OF SPECIFIC RADIOACTIVITY IN DIFFERENT SUBCELLULAR FRACTIONS OF DISRUPTED KREBS II ASCITES TUMOUR CELLS

Fraction	Protein		RNA	
	Percentage of total incorpn	Specific activity	Percentage of total incorpn	Specific activity
Nuclei	4	0·25	88	30
Mitochondria	24	0·90	2·3	0·6
Microsomes	33	1·05	4·5	0·3
Cell sap	39	0·60	5·2	1·8

Cells were incubated with either ^{14}C-orotic acid or ^{14}C-valine for 30 min at 37° in Earle's medium. Specific activities are expressed as μc/g protein and as μc/mmole RNA-phosphorus (data from Martin & Work, 1961).

The suggestion from these figures and from those given in Table 1 is that although nearly 90 per cent of the total RNA is found in the cytoplasm, nearly all the RNA synthesis takes place in the nucleus. In contrast, nearly all protein synthesis appears to occur in the cytoplasm.

THE SITE OF RNA SYNTHESIS

The suggestive nature of these results prompts us to pose our first major question for discussion. Is there synthesis of RNA in the cytoplasm or is all specific RNA synthesis intranuclear? We must emphasize the importance in this question of the word *specific*.

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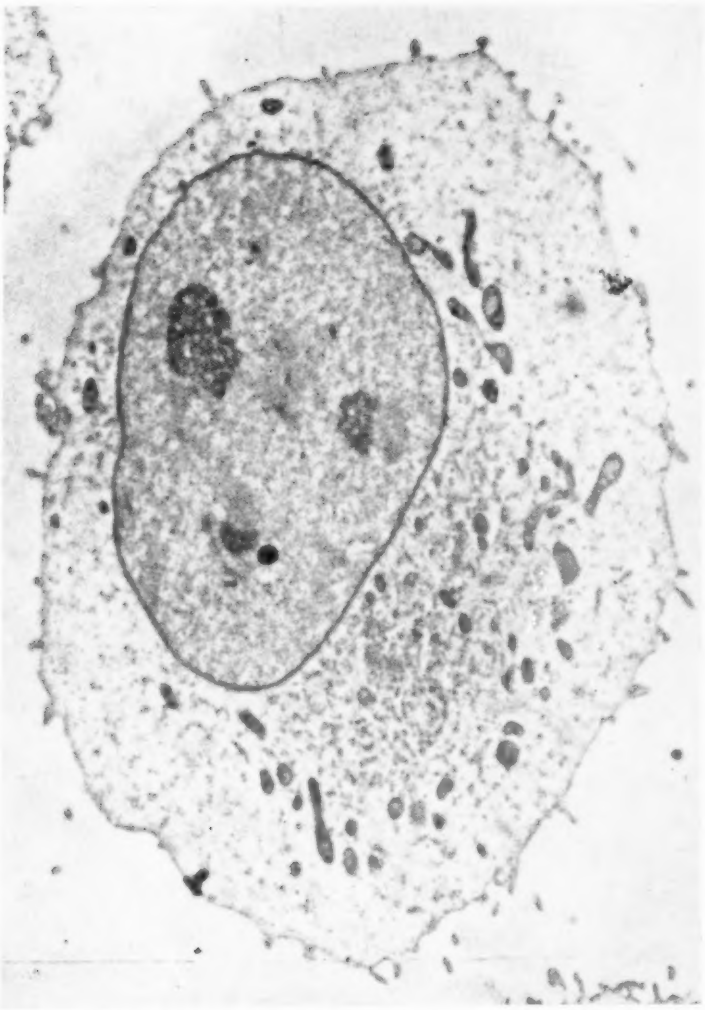
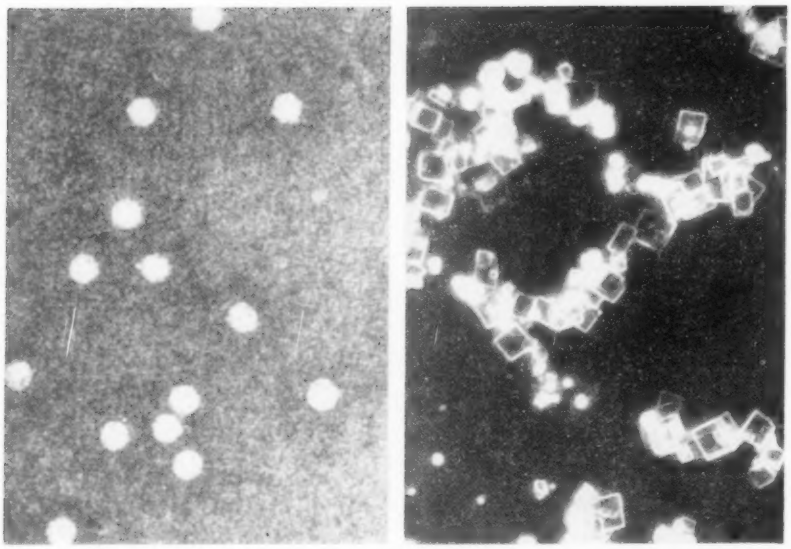


FIG. 1. Krebs II ascites tumour cell fixed in osmic acid. Ultra-thin section. Magnification × 7000.



(*A*) (*B*)

FIG. 2. Encephalomyocarditis virus. (*A*) Electron micrograph of phosphotungstate preparation. Magnification × 200,000. (*B*) Photograph of crystalline preparation of encephalomyocarditis virus. Magnification × 600.

The Krebs cell does not appear to be in any way unusual as regards RNA synthesis in the nucleus. Autoradiography has shown that, when various cells are incubated with radioactive precursors of RNA, the nucleus becomes much more rapidly labelled than the cytoplasm (Goldstein & Micou, 1959; Harris, 1959; Zalokar, 1959, 1960). Indirect methods such as autoradiography, which do not involve isolation of RNA from the subcellular components, are open to objection but nevertheless they do give a significant indication of the probable site of RNA synthesis which is in line with most other evidence. Thus, it has been shown that in liver (Bhargava *et al.*, 1958) and in pancreas (Siekevitz & Palade, 1959), the turnover of ribosomal RNA is low. Careful fractionation of a pancreatic homogenate after labelling of pancreas with ^{14}C-adenine gave the results shown in Table 3.

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TABLE 3—THE DISTRIBUTION OF RADIOACTIVITY IN RNA FROM SUBCELLULAR FRACTIONS OF GUINEA PIG PANCREAS 40 MIN AFTER INJECTION OF ^{14}C-ADENINE

Fraction	Specific activity of RNA
Attached ribosomes	40
Reticulum RNA	80
First post-microsomal fraction	60
Second post-microsomal fraction	230
Soluble supernatant	910

The cytoplasmic fractions were separated by differential centrifuging in sucrose (data from Siekevitz & Palade, 1959).

The high specific activity of the RNA of the soluble fraction clearly distinguishes this fraction from the particulate forms of RNA and indicates that this fraction may be synthesized independently of the bulk of the RNA.

Our second and supplementary question must therefore be: is soluble ribonucleic acid synthesized in the cytoplasm? Although the rapid labelling of cytoplasmic soluble RNA in the experiments of Siekevitz & Palade (Table 3) does suggest a cytoplasmic origin for SRNA the situation is complicated by the capacity of soluble RNA to add terminal adenine and cytosine. It has been shown that in cell-free preparations most of the labelling of soluble RNA is due to this terminal addition (Canellakis, 1957; Herbert, 1958; Hecht *et al.*, 1958). While Siekevitz & Palade did obtain non-terminal addition of adenine in their whole animal experiments, the existence of a system for terminal addition of adenine in the cytoplasm complicates the issue. Soluble RNA does, however, contain quite large quantities of pseudouridine (Dunn, 1959) and it might be possible to obtain more conclusive evidence as to the origin of soluble RNA with the aid of radioactive pseudouridine.

A supplementary line of evidence concerning the site of synthesis of particulate and soluble cytoplasmic RNA is provided by experiments on enucleated cells.

Prescott (1959) found that enucleated *Amoeba proteus* could not synthesize any RNA, while Naora *et al.* (1960) found that in enucleate *Acetabularia* there was no synthesis of RNA and that the RNA turnover was confined entirely to the chloroplasts, bodies not present in the cytoplasm of most cells. While negative evidence can never carry too much weight, these investigations certainly suggest that soluble RNA is not synthesized in the cytoplasm.

BIOCHEMICAL ANALYSIS OF VIRUS GROWTH

It has been apparent for some time now that a major impediment to progress in the definition of the site of RNA synthesis and also in attempts to relate RNA synthesis to protein synthesis is the absence of effective methods for the purification and characterization of specific RNA molecules. This difficulty can be largely overcome by making use of a suitable biological system. Viruses are synthesized within cells and it is reasonable to assume that synthesis of virus protein can be taken as a model for protein synthesis; and formation of viral RNA or viral DNA may similarly serve as a model for nucleic acid synthesis. Three possible systems are available for study, the bacterium bacteriophage system, the animal virus–animal cell system and the corresponding plant systems. In all these systems it is possible to purify both the protein and the nucleic acid in the form of free virus and in some of them it is possible to detect free nucleic acid by its infectivity. Much attention has already been devoted to bacteriophage but owing to the small size of the bacterial cell and its lack of a well-defined and isolable nucleus, little has been done to locate the site of synthesis of bacteriophage components, which are in any case complex and do not include RNA. Plant viruses also offer considerable technical difficulty since no satisfactory and uniform host cell suspension is available. A promising system, however, is provided by encephalomyocarditis virus growing in the Krebs II ascites tumour cell. As already mentioned, the host cell can be obtained in quantity as a uniform cell suspension and can be maintained in a simple synthetic medium. Hoskins & Sanders (1957) showed that after infection of Krebs cells with virus there was an eclipse period of about 4 hr and this was followed by a period of intracellular formation of new virus also lasting about 4 hr. A typical growth curve is shown in Fig. 1. The virus has recently been isolated and crystallized (Faulkner *et al.*, 1960). It contains about 70 per cent protein and 30 per cent RNA and probably has a subunit structure rather similar to turnip yellow mosaic virus (Faulkner *et al.*, 1961).

The virus can be readily assayed by plaque count methods (Sanders, 1957) and can be isolated on a micro scale by adsorption on to red cell membranes (Martin & Work, 1961). In addition infective RNA may be isolated from the host cell (Huppert & Sanders, 1958) and while there is at present no absolute proof that infective RNA is identical with virus RNA, it would be most surprising if this were not so.

During the past 4 years we have collaborated with Sanders and his colleagues in a study of the biochemistry of virus growth in suspensions of Krebs II cells. In a series of experiments, infected cells were disrupted at different times during the

virus growth cycle. The subcellular components were separated and each was assayed for haemagglutinin (virus protein). At no stage during the growth cycle was any significant quantity of haemagglutinin detected in the host cell nucleus. In other words synthesis of virus protein was cytoplasmic. We can go further than this and say that haemagglutinin was associated largely with the mitochondrial fraction and to a lesser extent with the microsomes. We do not at present know the significance of this association between mitochondria and haemagglutinin. It may be that as synthesis requires an abundant supply of ATP, new synthesis of protein can take place most readily on or near the mitochondria. We found also that completed virus (infective particle count) was associated with the mitochondrial fraction. Bellett & Burness (1960), working with Sanders, found, however, that during the early stages of the growth cycle infective RNA was associated almost entirely with the nucleus. This physical separation of specific RNA synthesis from protein synthesis requires that if the RNA is to act as template for synthesis of a specific protein it should be synthesized before the protein and should move from nucleus to cytoplasm before protein synthesis commences. It appears that both these requirements are fulfilled. By incubation of infected cells with ^{14}C-orotic acid and ^{14}C-valine it was possible to follow the time course of synthesis of the RNA and the protein which eventually appeared as complete virus. When this was done it was found that RNA synthesis preceded protein synthesis by about 45 min (Martin & Work, 1961). Moreover, when assays of infective RNA were made on all cell fractions throughout the growth cycle it was found that at about 4 hr after the start of the growth cycle infective RNA which had accumulated within the nucleus began to decrease, and at about the same time infectious virus particles began to be detected within the cytoplasm (Bellett & Burness, 1960). There is at present no clear indication as the form in which RNA is passed from nucleus to cytoplasm and experiments are in progress to determine this.

It has been possible, therefore, by use of the infected Krebs cell to establish rather firmly several points which could not be established in more normal systems. Firstly, specific RNA is synthesized in the nucleus before the synthesis of specific protein. Secondly, specific RNA must be passed into the cytoplasm before it can stimulate synthesis of specific protein. Thirdly, specific protein synthesis is associated with the mitochondrial fraction. We must emphasize, however, that this does not necessarily imply that virus protein synthesis takes place within mitochondria.

REQUIREMENTS FOR THE SYNTHESIS OF SPECIFIC RNA MOLECULES

The results of our own investigations and those of Sanders and his colleagues on encephalomyocarditis virus prompt us to ask our third question—why should the synthesis of specific RNA structures take place only within the nucleus? There is now considerable evidence that RNA can control protein synthesis, yet it is one of the striking facts of biology that all self-reproducing systems contain DNA as well as RNA. If RNA could be replicated without the intervention of DNA then during the course of evolution some biological system might have been

expected to dispense entirely with the need for DNA. These considerations prompted one of us to suggest earlier (Work, 1960) that synthesis of RNA requires the pre-formation of a mixed RNA–DNA double helix. It is now fairly certain from the work of Kornberg and his colleagues that the synthesis of specific DNA molecules from the four deoxyribonucleotide triphosphates can take place as a surface polymerization upon the primer DNA added to the enzyme system (Lehman *et al.*, 1959). Attempts to obtain similar RNA synthesis in cell-free preparations have recently been successful and in this case also DNA is required as primer (Hurwitz, 1959; Hurwitz *et al.*, 1960). The RNA molecule probably exists in a single stranded configuration (Littauer & Eisenberg, 1959; Littauer *et al.*, 1960; Hall & Doty, 1959), and it is not unreasonable to suggest that it must form a double stranded structure with DNA before it can act as a template for synthesis of new RNA molecules. Such double helical structures, part DNA part RNA, have been shown to be physically possible by Rich (1960).

It follows from these suggestions that in nucleated cells DNA synthesis and RNA synthesis might be mutually exclusive and that RNA synthesis might be expected to stop during periods of DNA synthesis. In support of this idea it should be noted that Prescott (1960), in a study of synchronously dividing *Tetrahymena*, found that while protein synthesis was continuous, RNA synthesis and DNA synthesis were mutually exclusive.

THE PHYSICAL STATE OF RNA IN THE CYTOPLASM

We have already emphasized that although most of the RNA synthesis takes place in the nucleus, around 90 per cent of the total RNA is found in the cytoplasm. At least four distinct RNA fractions may be recognized in the cytoplasm of various cells. The most important, at least quantitatively, is the ribosomal RNA but in cells with a well-developed endoplasmic reticulum the amount of RNA in the reticulum fraction may approach that in the ribosomes (Moule *et al.*, 1960). The RNA of mitochondria represents a much smaller fraction of the total cellular RNA but it is undoubtedly an important part of the mitochondrial complex and is probably associated with protein synthesis in this organelle (Roodyn *et al.*, 1961). So far as is known the base compositions of ribosomal RNA, reticulum RNA and mitochondrial RNA do not differ greatly, and in this respect all three can be distinguished from the soluble RNA of the cytoplasm (Dunn, 1959; Scholtissek, 1959; Siekevitz & Palade, 1959; de Lamirande *et al.*, 1959; Kit, 1960; Dunn *et al.*, 1960).

It is now generally agreed that soluble RNA has a low molecular weight and that it can be chemically purified (Brown *et al.*, 1959; Zamecnik *et al.*, 1960). As new data on soluble RNA will be presented later in this symposium we shall not elaborate either upon its properties or upon its importance. Attention should be focused, however, on the lack of information and even lack of agreement concerning the other forms of cytoplasmic RNA. It is still quite uncertain whether the RNA of the ribosomes has a mol. wt. around 100,000 or over 1,000,000 or whether there is a large range of mol. wts. (Gierer, 1958; Hall & Doty, 1959; Bloemendal *et al.*,

1960; Cox & Littauer, 1961). This difficulty arises partly as a result of the association of ribonuclease with the ribosomes and with the resultant tendency for RNA to be degraded during isolation of particles. This degradation can probably be overcome by the use of phenol to separate the protein and RNA of ribosomes. Even when phenol was used, however, Bloemendal *et al.* (1960) obtained at least three distinct types of RNA. One of these appeared to be an acceptor of nucleotides from soluble RNA whereas the others were inactive. There is, however, a serious difficulty in interpreting physico-chemical studies on RNA obtained from ribosomes. It has been shown clearly by the investigations of Siekevitz & Palade (1959) that cells contain both attached ribosomes and free ribosomes and that these ribosomes have different metabolic turnover rates. Thus any study made on either RNA or protein from ribosomes is a study on material obtained from a heterogeneous collection of particles and the apparent heterogeneity of any extract may reflect this.

So far as we are aware virtually nothing is known concerning the physical properties of mitochondrial RNA. It is also unknown at present whether reticulum RNA has any resemblance to ribosomal RNA although in some cells at least it may be quantitatively equal to the ribosomal RNA. These are obvious and important gaps in our present knowledge of the cytoplasm.

THE ORIGIN OF RIBOSOMES

Four lines of evidence can be quoted to support the view that ribosomes are formed in the nucleus and passed as ribonucleoprotein particles into the cytoplasm. In our view none of these lines of evidence is entirely convincing. Electron micrographs do show ribosome-like structures within the nucleus (Porter, 1954) and the nuclear membrane contains pores large enough to allow passage of such structures (Watson, 1954). It is likely, however, that high molecular weight RNA will form ribosome-like structures spontaneously in the presence of any basic protein and the existence of such structures within the nucleus cannot be accepted as evidence that cytoplasmic ribosomes originated as nuclear ribosomes. A second argument has been that ribosome-like structures can be extracted from isolated nuclei (Frenster *et al.*, 1960). A third line of evidence adduced in favour of the nuclear origin of ribosomes is the resemblance between the amino acid composition of ribosomal protein and nuclear histone (Ts'o *et al.*, 1958; Crampton & Peterman, 1959) but this again is indirect evidence. The last and perhaps the most convincing source of evidence is electron microscopy of thin sections of cells. These sections show nuclear pores large enough to permit passage of ribosomes and the appearance of ribosomes passing through these pores. Unfortunately evidence obtained in this way is entirely static and cannot show that ribosomes originate within the nucleus (Bonner, 1958).

While all this evidence is certainly suggestive, it would seem wise at present to reserve judgement as to whether ribosomes are formed entirely within the nucleus. This reservation is made partly because protein turnover within the nucleus is known to be low, partly because electron micrographs of sections of liver prepared

from starved and re-fed animals indicate that regeneration of ribosomes occurs mainly around the mitochondria (Bernhard & Rouiller, 1956) and partly because we ourselves have found that encephalomyocarditis virus protein is synthesized in the cytoplasm although the necessary RNA appears to be supplied by the nucleus. We are of course making the assumption that virus formation is an analogous process to ribosome formation and this assumption may not be entirely correct.

While it must be admitted, therefore, that there is no unequivocal proof of the physical state of RNA passed from nucleus to cytoplasm, experiments with isolated rat liver nuclei suggest that RNA can pass from the nucleus into the cytoplasm as free RNA (Scholtissek & Potter, 1960). This fraction of the nuclear RNA has a particularly high turnover rate. Martin (unpublished) has found also that in Krebs II cells a small fraction of the nuclear RNA has an exceptionally high turnover rate and that this fraction is readily released from isolated nuclei. Experiments are in progress with cells infected by encephalomyocarditis virus to try to demonstrate the presence of infective RNA in this fraction but so far this has not been achieved.

It must be clear from this discussion that remarkably little is known as yet regarding either the origin or the heterogeneity of cytoplasmic RNA. One of the major difficulties is the absence of effective methods for the fractionation of either ribosomes or of high mol. wt. RNA. Quantitative measurements made on material extracted from a heterogeneous collection of particles cannot be effectively interpreted and one is inevitably compelled to investigate model systems such as that provided by the virus-infected cell where at least the RNA has a measurable biological activity. Recently, some attempts have been made to overcome these difficulties by study of events within single cells. Although these methods have serious technical limitations and cannot prove the origin of any particular type of RNA molecule, results are consistent with the assumption that RNA is synthesized most rapidly in close association with the DNA of the nucleus. It may be synthesized also within the nucleolus and both types of RNA may be passed to the cytoplasm (Perry, 1960; Amano & Leblond, 1960).

THE ORIGIN OF MITOCHONDRIA

The available evidence on the origin of mitochondria is both scanty and contradictory.

Recent electron micrographs suggest that mitochondria may arise by budding from the nuclear membrane (Hoffman & Grigg, 1958) but other equally convincing micrographs show mitochondria in the process of binary fission (Fawcett, 1955). Any theory as to the origin of mitochondria must take into account the presence in these organelles of proteins not found elsewhere in the cytoplasm; proteins with defined structures which are probably synthesized *in situ* but which nevertheless are almost certainly under genetic control. This would require, according to current hypotheses, that mitochondrial RNA like microsomal RNA should originate within the nucleus.

It has been suggested by Rendi (1959) that mitochondria contain ribosome-like structures. While this suggestion has attractions as a unifying concept it is not

entirely consistent with current evidence. Many electron micrographs of ultra-thin sections of mitochondria have been published but these do not show ribosomes attached internally to the mitochondrial membranes. In this laboratory, we have disrupted mitochondria by mechanical means and by detergents. In this way an RNA-rich fraction can be obtained and this fraction contains protein which has a high turnover rate but the fraction does not have much analytical resemblance to ribosomes (Roodyn *et al.*, 1961). It must be admitted, therefore, that at present we cannot do much more than guess at the origin of mitochondria and the mechanisms whereby they seem able to synthesize specific proteins.

THE CELL SAP

There is quite general agreement that proteins cannot be synthesized by a cell sap which has been freed of subcellular organelles by high-speed centrifuging. In intact cells, also, it has been shown that there is a significant delay between addition of radioactive tracer to the incubation medium and the appearance of radioactive protein in the cell sap, whereas there is almost no delay in the appearance of radioactive protein in the subcellular organelles (Siekevitz, 1959). Because of these results, it was particularly surprising to find that Kalf & Simpson (1959) were able to extract from disrupted mitochondria (heart sarcosomes) a soluble system which was capable of incorporation of radioactive amino acid into protein, this incorporation being inhibited by ribonuclease. It would be interesting to know, therefore, whether anyone has attempted to obtain an active soluble system by the sonic disruption of ribosomes. It may be that the association of ribonuclease with the ribosome fraction would render such experiment abortive but it now seems that, in *Esch. coli* at least, ribonuclease is associated with only one class of ribosome (Elson, 1961) so that the experiment is not foredoomed to failure.

The assumption that the RNA of the ribosomes is the complete template controlling the specificity of protein synthesis would certainly accord with present experience. In the cell-free preparations so far examined, the biological origin of the soluble fraction of the incubation mixture does not influence the nature of the protein synthesized and this is governed by the nature of the ribosome (Lamfrom, 1960; Campbell & Kernot, 1959). It has been found by Ogata (1961), however, that soluble RNA may have a coenzyme-like function. It may control either the activity or the specificity (results reported do not permit distinction between these possibilities) of the amino acid activating enzymes. Gros (1961) has shown also that in *Esch. coli* a rather low mol. wt. RNA fraction is concerned in the control of protein synthesis. These observations inevitably create doubt as to whether the specificity of protein synthesis is governed entirely by a specific template resident in the ribosome.

CONCLUSION

In reviewing present knowledge of protein and RNA synthesis we have deliberately avoided detailed discussion of points which are likely to be covered by other contributors to this symposium. If rather much attention has been devoted

to RNA synthesis in the nucleus this is because cytoplasmic function cannot be considered in isolation. Emphasis must be placed upon the need for more information on both the physical and chemical aspects of RNA structure and particularly upon the need for improved methods of fractionation of RNA. Meantime there is no doubt that a virus system such as that which we have described offers great advantages in the study of both RNA synthesis and protein synthesis.

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COMPARATIVE BIOCHEMISTRY OF PARASITIC HELMINTHS*

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While biochemical patterns common to many other forms of life can be demonstrated in parasitic helminths, this group of organisms also illustrates biochemical diversity at a variety of levels. Enzymes catalyzing the same reaction in the parasitic trematode *Schistosoma mansoni* and its mammalian host can be distinguished from each other (a) by immunological procedures, (b) by their affinities for their substrates and co-factors and by their pH optima, (c) by their substrate specificities, and (d) by their sensitivity to inhibitors. Such specificities can account also for the fact that formation of cyclic 3,5-AMP and of phosphorylase activity is enhanced by two different activators, epinephrine in host tissues (liver, heart, skeletal muscle), and 5-hydroxytryptamine in the liver fluke, *Fasiciola hepatica*.

Hitherto, the only unequivocal evidence for the conversion of fat to carbohydrate in animal tissues has been provided by studies with *Ascaris* eggs. The muscle of adult *Ascaris* contains a particulate succinic and DPNH oxidizing system which operates without the participation of the cytochrome system and whose terminal electron is a flavoprotein. Anaerobically, this preparation catalyzes the reduction of fumarate to succinate by DPNH at a rapid rate. Under the essentially anaerobic conditions of the habitat of *Ascaris*, this system can serve as a mechanism for the reoxidation of DPNH. Another biochemical characteristic distinguishing *Ascaris* from vertebrates and from other species is the formation of branched chain 5-carbon acids produced by condensation of the carboxyl carbon of acetate with the *a*-carbon of propionate.

These examples tend to emphasize the need for recognizing and defining the nature of specific biochemical differences beneath an apparent uniformity of biochemical characteristics among all forms of life.

The occurrence of the Embden–Meyerhof scheme of phosphorylating glycolysis, of the pentose phosphate pathway, of the Krebs tricarboxylic acid cycle, of the cytochrome system and of many other biochemical processes has been demonstrated in vertebrate tissues, in unicellular organisms as well as in plants. Another feature common to most, if not to all, living cells is the reactions leading to the production of ATP as a source of energy. Because of these common patterns, there has been a tendency to emphasize these similarities and to overlook the possibility of biochemical differences. In fact, it has been stated that comparative biochemistry should be concerned primarily with metabolic reactions common to all forms of life (Kluyver & van Niel, 1956). Originally such a point of view has been a productive one because it has stimulated efforts to uncover reaction sequences and basic mechanisms by which energy is supplied to living cells. However, once this had been accomplished, a rigid concept of the "unity of biochemistry" fails to take into account that species differences are in some manner associated with biochemical variabilities which, as pointed out by Dixon & Webb (1958), ultimately are due to qualitative or quantitative enzymatic differences. These may be the result of the complete absence of one or of several enzymes from a particular species or organism. Quantitative differences may be reflected in the weak activity of one enzyme, as contrasted with the high activity of another enzyme

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acting on the same substrate. Such variations can result in a diversion of the substrate into another metabolic pathway and thus yield a different metabolic product. Furthermore, even if given metabolic pathways or reactions are identical in two different species, the enzymes catalyzing these reactions may differ from each other.

Although our knowledge about the biochemistry of mammalian tissues and of bacteria is far more advanced than about that of parasitic helminths, observations made with members of the latter group of organisms can serve as examples for biochemical differentiation at a variety of levels.

SCHISTOSOME ENZYMES

THE habitat of the blood fluke *Schistosoma nansoni*, a parasitic trematode, is the portal-mesenteric venous system which has a relatively high oxygen tension (Blalock & Mason, 1936; Engel *et al.*, 1944). Yet, this organism depends almost entirely on the anaerobic utilization of carbohydrate as a source of energy for survival and reproduction (Bueding, 1950; Bueding & Peters, 1951; Bueding *et al.*, 1953). In this parasite carbohydrate utilization proceeds at a high rate, is not affected by aerobic conditions (Bueding, 1950), and consists in the quantitative conversion of glucose to lactic acid via the Embden–Meyerhof scheme of phosphorylating glycolysis (Mansour & Bueding, 1953, 1954; Bueding & Mackinnon, 1955a, b; Bueding & Mansour, 1957). While in schistosomes the classical reaction sequences of this pathway are identical with those discovered in many types of species and cells, differences between the glycolytic enzymes of schistosomes and those of their mammalian host have been demonstrated on at least four different levels.

(1) While the kinetic properties of the phosphoglucose isomerases of schistosomes and of their mammalian host are identical, they can be distinguished from each other by the use of immunological procedures (Bueding & Mackinnon, 1955b). Sera from roosters which had been immunized against phosphoglucose isomerase of the parasite markedly inhibit the activity of this enzyme, but do not affect the activity of the rabbit isomerase. Since the activities of other glycolytic enzymes of schistosomes are not inhibited by the same antiserum (Bueding & Mackinnon, 1955b), it is evident that the latter reacts with sites specific for a single enzyme rather than with groupings common to schistosome proteins. Similar specificities have been demonstrated with an antiserum against lactic dehydrogenase of *Schistosoma mansoni* (Bueding, 1952). In this case, pre-incubation of schistosome lactic dehydrogenase with its coenzyme, DPNH, affords almost complete protection from the inhibitory effects of its anti-serum. Therefore, it appears that at least one point of attachment of the antibody is localized either at or near the active center of the enzyme.

(2) Protection of schistosome lactic dehydrogenase from its antiserum by DPNH does not prove necessarily that the active centres, rather than some other constituents, or the lactic dehydrogenases of the parasite and of the host differ from each other. However, when differences in kinetics, in pH optima, as well as in substrate and cofactor requirements of homologous* enzymes are observed also,

* In this paper the term "homologous" is used to designate enzymes which catalyze the same reaction in different species.

the question may be raised whether the active centers of these two enzymes are identical. The occurrence of such differences has been reported for several glycolytic enzymes catalyzing the same reactions in *Schistosoma mansoni* and in mammalian muscle. For example, the pH optimum for lactic dehydrogenase of schistosomes is much lower than that for rabbit muscle lactic dehydrogenase (Mansour & Bueding, 1953). Furthermore, the dissociation constant of the latter for pyruvate is 12 times lower than that for the schistosome enzyme (Timms & Bueding, 1959). Similar kinetic differences have been observed for the phosphofructokinases of schistosomes and of rabbit muscle (Bueding & Mansour, 1957), and for specific acetyl-cholinesterases of *Schistosoma mansoni* and mammalian brain (Henion *et al.*, 1955).

(3) Probably an even greater degree of difference in the nature of the active center must prevail when variations in the substrate specificity are encountered among homologous enzymes. Particulate mammalian brain hexokinase and soluble yeast hexokinase catalyze the phosphorylation of a variety of hexoses (Crane & Sols, 1953; Berger *et al.*, 1946), while in schistosomes at least four different hexokinases are present, each one of which is specific for a single hexose (glucose, fructose, mannose and glucosamine) (Bueding & Mackinnon, 1955; Bueding *et al.*, 1954). Schistosomes also contain a protease which has a high specificity towards hemoglobin or globin and which does not degrade other proteins (Timms & Bueding, 1959). Since schistosomes ingest red cells, their protease, by catalyzing the digestion of hemoglobin, may provide a supply of peptides or amino acids to the parasite. This is suggested also by the observation that a synthetic medium containing amino acids in the same proportions as in the globin molecule supports the survival of *Schistosoma mansoni in vitro*, while other amino mixtures fail to do so (Timms & Bueding, 1959).

(4) Differential susceptibility to inhibitors is another characteristic distinguishing a homologous enzyme of the parasite from that of the host. In contrast to mammalian phosphofructokinase, the enzyme catalyzing the phosphorylation of fructose-6-phosphate by ATP is inhibited by low concentrations of organic antimonials (potassium antimony tartrate, stibophen) (Mansour & Bueding, 1954). The chemotherapeutic activity of these compounds in the treatment of schistosomiases can be accounted for by the inhibitory effect of antimonials on the activity of phosphofructokinase of *Schistosoma mansoni* because (a) the rate of glycolysis of the parasite is determined by the rate of the phosphofructokinase reaction; (b) glycolysis supplies the major source of energy for the organism, and (c) exposure of schistosomes to subeffective concentrations of antimonials *in vitro* or within their host results in the accumulation of the substrate (fructose-6-phosphate) and in a decrease of the product (fructose-1, 6-diphosphate) of the phosphofructokinase reaction within the parasite (Bueding & Mansour, 1957). This drug-enzyme interaction indicates that differential sensitivities of homologous enzymes might provide opportunities to develop antiparasitic agents through selective inhibition of the activity of an enzyme catalyzing an essential rate-limiting reaction of the invading organism.

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The foregoing examples demonstrate the existence of differences in the nature of enzymes catalyzing the same reactions in a parasitic helminth and in its mammalian host. In some cases homologous enzymes can be distinguished only by the use of specific antibodies. In other instances such enzymes differ from each other also with regard to their affinities for their substrates, the effect of the hydrogen ion concentration on their activities and in their cofactor requirements. In other cases, differences in substrate specificities as well as in kinetics are encountered. Finally, the functional integrity of an enzyme of the parasite may be interfered with by certain inhibitors which have no effect on the homologous enzyme of the host. Therefore, enzymes not only are endowed with substrate specificities, but also may exhibit species specificities.

BIOCHEMICAL EFFECTS OF 5-HYDROXYTRYPTAMINE ON *FASCIOLA HEPATICA*

Some metabolic characteristics of the liver fluke *Fasciola hepatica* supply an example for another pattern of biochemical difference. The major fermentation products of this parasite are propionic and acetic acids; only relatively small amounts of lactic acid are formed by this organism during incubation in a glucose-containing Ringer solution (Mansour, 1959a). On the other hand, low concentrations of 5-hydroxytryptamine (5-HT; "serotonin") produce a marked increase in lactate production (Mansour, 1959b). This is associated with a stimulation of the muscular activity of the worm (Mansour, 1957), of glycogenolysis (Mansour, 1959b), and of phosphorylase activity (Mansour *et al.*, 1960). 5-HT increases the formation of cyclic adenosine-3,5′ phosphate (3,5-AMP) by particulate preparations of *Fasciola hepatica* (Mansour *et al.*, 1960). As demonstrated by Sutherland *et al.* (Mansour *et al.*, 1960; Sutherland & Rall, 1958) 3,5-AMP promotes the accumulation of active phosphorylase in dog liver. This is brought about by the transfer of phosphate from ATP to inactive phosphorylase and is catalyzed by a kinase (Rall *et al.*, 1956). Similarly, 3,5-AMP increases the formation of active phosphorylase in mammalian heart (Sutherland & Rall, 1948) and skeletal muscles (Krebs *et al.*, 1959). In these three mammalian tissues, formation of 3,5-AMP is stimulated by epinephrine (Sutherland & Rall, 1958; Murad *et al.*, 1960), while 5-HT has no effect (Sutherland & Rall, 1960). By contrast, in *Fasciola hepatica*, neither the activation of 3,5-AMP formation nor the stimulation of the muscular activity and of glycogenolysis produced by 5-HT is shared by epinephrine (Mansour, 1959b, 1957; Mansour *et al.*, 1960). Thus, 3,5-AMP has an activating effect on phosphorylase in the tissues of both the host and the parasite, but two distinct compounds function as activators of 3,5-AMP formation—epinephrine in many host tissues, 5-HT in the parasite.

METABOLISM OF *ASCARIS*

The metabolism of *Ascaris lumbricoides* illustrates still other aspects of biochemical variability of the parasite from the host. In mammalian tissues, the net

formation of carbohydrate from lipids could not be demonstrated (Lifson *et al.*, 1948). On the other hand, Passey & Fairbairn (1957) have found that when *Ascaris* eggs develop to the infective stage (from the 10th to the 25th day of their development) there is an increase in carbohydrate which is accounted for by the decrease in non-volatile fatty acids. In a carefully documented study, these authors excluded any other source for the synthesis of carbohydrate. These observations provide the first unequivocal evidence for the conversion of fat to carbohydrate in animal tissues.

Although the habitat of adult *Ascaris*, the intestinal tract, is essentially anaerobic, this worm takes up oxygen in an atmosphere containing a relatively high concentration of this gas (Fairbairn, 1957). In an attempt to analyze this phenomenon, Harnisch (1937) has found that after repeated washings the respiration of minced *Ascaris* tissues is abolished, but that oxygen consumption is restored by addition of the perienteric fluid of the worm. Confirming these observations, it has been found that the ability of perienteric fluid to stimulate the oxygen uptake of washed *Ascaris* muscle preparations is not altered by heat, but is lost on dialysis (Bueding & Charms, 1952). Subsequently, this material has been identified as succinic acid (Bueding & Farrow, 1956). Therefore, the oxygen uptake of *Ascaris*, at least in part, can be ascribed to the oxidation of succinate which is present in rather high concentrations (5 to 12 M/ml) in the perienteric fluid of the parasite (Bueding & Farrow, 1956). These findings suggest that *Ascaris* tissues contain a succinoxidase system. Among the tissues of the parasite, muscle has the highest activity.

In spite of the ability of *Ascaris* muscle to oxidise succinate, this tissue contains no measurable cytochrome *c* or cytochrome oxidase activities (Bueding & Charms, 1952). Furthermore, H_2O_2 is formed during the oxidation of succinate (Laser, 1944), and in 100% oxygen succinate oxidation is considerably higher than in air (Bueding & Charms, 1952). Therefore, the absence of significant cytochrome *c* and cytochrome oxidase activities from *Ascaris* muscle, the production of H_2O_2 rather than of water during succinate oxidation, and the dependence on a high oxygen tension for optimal activity indicate that, in contrast to the mammalian system, the terminal oxidase of *Ascaris* muscle succinoxidase is a flavoprotein. This is borne out also by the fact that neither cyanide (Laser, 1944) nor antimycin *a* (Bueding *et al.*, 1955) inhibits the activity of the *Ascaris* succinoxidase system. Another difference between the mammalian and *Ascaris* succinoxidases consists in the fact that the *Ascaris* system is stimulated by Mn (Bueding & Charms, 1952; Kmetec & Bueding, 1961). As the mammalian system, the succinoxidase of *Ascaris* muscle is particulate (Bueding & Charms, 1952). It can be purified by differential centrifugation, solubilization with deoxycholate, and subsequent fractionation with ammonium sulfate, yielding approximately a 45-fold purification over the original muscle homogenate (Kmetec & Bueding, 1961). In addition to succinoxidase activity, the purified *Ascaris* system also exhibits DPNH oxidase activity (Kmetec & Bueding, 1961). The latter, as the succinoxidase system, is inhibited by EDTA, and its activity is restored by excess Mn^{2+}. Furthermore,

DPNH oxidase activity also is highly dependent on the oxygen tension. Finally, during the oxidation of both these substrates by the purified *Ascaris* system, H_2O_2 is formed (Kmetec & Bueding, 1961). These properties suggest that both the succinate and DPNH oxidases of *Ascaris* particles react with atmospheric oxygen by means of a common flavin-containing terminal oxidase.

The activity of *Ascaris* succinoxidase preparations is highest with phenazine methosulfate, lower with methylene blue, and lowest with oxygen as an electron acceptor (Bueding *et al.*, 1955; Kmetec & Bueding, 1961). Therefore, at least three different reactions are involved in the transport of electrons from succinate to atmospheric oxygen. Various fractionation procedures have supplied more direct evidence for the occurrence of these components in this system (Kmetec & Bueding, 1961). The dehydrogenase can be dissociated from the remainder of the electron transport system by treatment with acetone; after treatment of *Ascaris* muscle succinoxidase with lipase, the activity with oxygen is markedly reduced, while the ability of the preparation to oxidize succinate in the presence of methylene blue or phenazine methosulfate remains unimpaired.

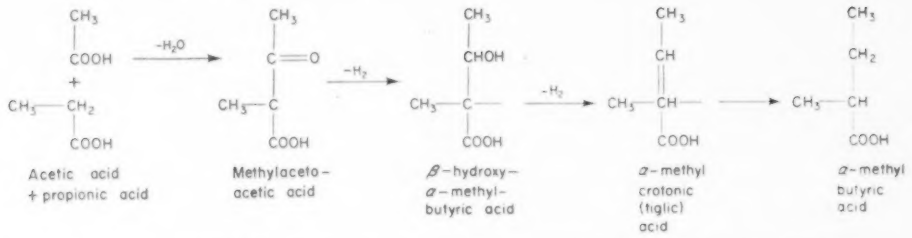
In contrast to succinate, lactate does not accumulate in media in which this organism has been incubated, despite the occurrence in *Ascaris* muscle extracts of phosphorylating glycolysis according to the Embden–Meyerhof scheme (Bueding & Yale, 1951). This raises the question of how DPNH, formed as a result of the oxidation of glyceraldehyde-3-phosphate, is oxidized under anaerobic conditions. Using ^{14}C-labeled CO_2 and also distinctly ^{14}C-labeled lactate, Saz & Vidrine (1959) have demonstrated that muscle strips of *Ascaris* produce succinate by CO_2 fixation into pyruvate (probably through the action of a malic enzyme (Saz & Hubbard, 1957)). Also, the occurrence of a fumarase in *Ascaris* muscle has been demonstrated (Saz & Hubbard, 1957). These findings suggest that in this tissue succinate may be formed through a reversal of the succinoxidase reaction. Indeed, this can be brought about by the particulate succinic-DPNH oxidase system because incubation of this preparation with both DPNH and fumarate results in the formation of succinate (Kmetec & Bueding, 1961). The rate of fumarate reduction by this system proceeds at a rapid rate—in fact, this rate is more rapid than the oxidation of succinate or DPNH by this system in the presence of methylene blue. The reduction of fumarate by DPNH, catalyzed by the *Ascaris* system, suggests that electrons from DPNH may be directed either to O_2 or, in the absence of this gas, to fumarate via a transport system which can serve as an electron acceptor from succinate or as an electron donor to fumarate.

Thus, in the essentially anaerobic physiological habitat of *Ascaris*, the formation of succinate results in the oxidation of reduced DPN, thus providing a means of generating oxidized DPN which becomes available again for glycolysis. In addition, succinate production in *Ascaris* may serve some other function. It has been observed that aerobically the DPN-linked oxidation of pyruvate by particles of *Ascaris* muscle is associated with phosphorylations. Under these conditions the P:O ratio approaches 1. It would appear that phosphorylation occurs within the electron transport system during the dehydrogenation of DPNH by flavoproteins

at a site preceding the terminal oxidase because, in contrast to DPNH, succinate oxidation is not associated with phosphorylation (Chin & Bueding, 1954); therefore, there are no sites of phosphorylation along the electron transport chain from succinate to oxygen and also along that portion of the chain which is used in common with DPNH. Furthermore, phosphorylations are inhibited when DPNH and succinate are oxidized simultaneously (Chin & Bueding, 1954). This probably is due to a suppression of DPNH oxidation because of the competition of the two substrates for the same terminal oxidase. It is possible that this type of phosphorylation which is uncoupled by dinitrophenol may occur, not only in the presence of oxygen, but also anaerobically during the transfer of electrons from DPNH to fumarate. It should be noted that the reversible paralysis of *Ascaris*, produced by the anthelmintic drug piperazine, is associated with a very marked reduction in the formation of succinate by the parasite (Bueding *et al.*, 1959); thus, it appears that succinate production supplies energy for muscular contraction of the worm; this energy could be provided by a reduction of fumarate by DPNH coupled with phosphorylation. In this manner, the observed succinate formation by *Ascaris* in its anaerobic habitat not only would provide a means for reoxidizing DPNH but also would generate energy in the form of ATP. It remains to be established whether anaerobic phosphorylations via reduction of fumarate by DPNH constitute a pattern for the supply of energy not only to *Ascaris* but also to other helminths which depend on anaerobic metabolism. Since CO_2 fixation is a pre-requisite for the anaerobic production of fumarate from pyruvate, it is of interest that CO_2 is essential for maintaining the functional integrity of the whipworm *Trichuris vulpis* (Bueding *et al.*, 1961), that succinate production via CO_2 fixation has been demonstrated in the roundworm *H. gallinae* (Fairbairn, 1954), and that carbohydrate metabolism of the tapeworm *H. diminuta* is stimulated markedly by atmospheric CO_2 (Fairbairn *et al.*, personal communication). As in the case of *Ascaris*, succinate is a fermentation product of these three other helminths (Bueding *et al.*, 1961; Fairbairn, 1954; Fairbairn *et al.*, personal communication). These mechanisms may not be limited to helminths because CO_2 is known to be required also by anaerobic succinate-forming bacteria (Ajl & Werkman, 1948; Hungate, 1950; Griffin & Racker, 1956; Gill & King, 1958; Larson *et al.*, 1959) and protozoa (Lwoff, 1951), and because anaerobic dinitrophenol-uncoupled phosphorylations have been observed in bakers' yeast (Meyerhof & Fiala, 1950).

Besides succinate, *Ascaris* produces large quantities of volatile acids among which acetic (Bueding & Yale, 1951), propionic (Bueding & Yale, 1951), and *n*-valeric acid (Bueding, 1953), as well as two branched chain 5-carbon acids, α-methylbutyric (Bueding & Yale, 1951) and α-methylcrotonic (tiglic) (Bueding, 1953) acids, have been identified. Regarding the mechanism of the formation of these acids, Saz *et al.* (Saz & Vidrine, 1959; Saz & Weil, 1960) have shown that in *Ascaris* muscle succinate is decarboxylated, resulting in the formation of propionate, and that in this tissue the branched chain carbon skeletons of tiglic and α-methylbutyric acids are produced by a condensation of the carboxyl carbon of acetate

with the α-carbon of propionate:



The above postulated sequence of intermediate reactions leading to the production of tiglic and α-methylbutyric acid has not been established experimentally but is consistent with the observed type of condensation between acetate and propionate. It is of interest that recently similar dimerizations of two carboxylic acids have been demonstrated in two bacteria. Gastambide-Odier & Lederer (1959) have reported that in *Corynebacteria* one palmitic acid molecule condenses with the α-carbon of another palmitic acid molecule which, after reduction, results in the formation of the branched chain acid, corynnemycolic acid. Furthermore, Corcoran *et al.* (1960) found that the branched side-chains of the antibiotic erythromycin are synthesized by condensations of carboxyl carbons of one propionate with the α-carbons of another propionate molecule.

CONCLUSIONS

The results of biochemical studies carried out with parasitic helminths support the views of Florkin & Mason (1960) that biochemical diversity must receive as much attention as biochemical unity. Undue emphasis on the latter will lead to a distorted picture. Thus, comparative biochemistry should be concerned with the nature of variability from patterns common to many forms of life and in this manner should contribute to a better understanding of biochemical evolution and adaptation.

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EVOLUTION OF THE BIOLOGICAL FIXATION OF NITROGEN

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THE outstanding achievements of contemporary chemistry and biology have done more than define paths for the further development of a number of special sciences. These advances are also changing our conceptions on the origin and development of life on this planet. The latest conceptions of astronomy, geology, geophysics, geochemistry and synthetic chemistry have made it possible to offer new solutions for a number of problems connected with the appearance and evolution of living beings on the earth. An excellent illustration of this possibility is afforded by the success of the special International Symposium in the U.S.S.R. on the origin of life on the earth. The advances in this field have brought it about that some of the conceptions previously shared by many outstanding scientists have now been completely abandoned. More particularly, this applies to the assertion that chemosynthesizing bacteria developed on the earth before heterotrophic micro-organisms. Today, this view has practically no supporters, as the abiogenic evolution of organic substances on the earth readily explains the original appearance of heterotropic bacteria. As comparative biochemistry develops, the compiling of schemas for the evolution of certain functional features of micro-organisms will undoubtedly become an actual possibility. The several considerations presented below on the evolution of nitrogen fixation can be regarded merely as a modest attempt to approach this problem in the light of the evidence now available on the biology and biochemistry of nitrogen-fixing micro-organisms.

THE SPREAD OF THE POWER TO FIX NITROGEN AMONG LIVING ORGANISMS

The history of the discovery of the power of micro-organisms to fix nitrogen is somewhat unique. The discoveries which determined the first stage in the development of this problem were made in a comparatively short space of time—13 years. In 1888 Hellriegell & Wilfahrt established the fact that nodule bacteria could assimilate atmospheric nitrogen; 5 years later, in 1893, Vinogradskii discovered the first free-living anaerobic bacteria, *Clostridium pasteurianum*, which fixed nitrogen; and in 1901 Beijerinck isolated the aerobic nitrogen-fixing micro-organism, *Azotobacter*. For a period of 40 years all investigations on nitrogen fixation were connected with study of the ecology, physiology and biochemistry of these three kinds of micro-organism or related forms.

The second period in the development of this problem started comparatively recently. It had several characteristic features.

It was first proved that the power to fix atmospheric nitrogen was much more widespread among lower plants than had previously been thought. It was found that many blue-green algae, and particularly representatives of the genera *Nostoc*, *Anabaena*, *Mastigocladium* and some others could be classed as nitrogen-fixers. It is difficult to overestimate the importance of this fact for an understanding of the nitrogen cycle in nature when we consider the wide distribution of these algae in waters and in some soils. Even more unexpected was the discovery of the power to fix nitrogen in various photosynthesizing bacteria (*Rhodospirillum*, *Chromatium*, *Chlorobium*, etc.).

The number of plant organisms definitely proved to be able to fix nitrogen has increased sharply in recent times.

Secondly, another feature of the new stage in the development of our conceptions on nitrogen fixation was the investigation of the process by means of nitrogen isotopes. These investigations have yielded very valuable results.

The investigation of nitrogen fixation at subcellular level must be regarded as the third feature of this period. Acellular fixation is now completely proved and this has made it possible to carry out enzyme investigations from a comparative standpoint (Wilson & Burris, 1960).

At the present time, therefore, the nitrogen-fixing organisms include representatives of very different taxonomic groups—spore-bearing bacteria, non-sporing bacteria, spirilla and blue-green algae. Parallel with the discovery of new micro-organisms which fixed nitrogen, there was repeated testing of the power of plants and animals cells to assimilate atmospheric nitrogen. These investigations merely served to confirm the fact established earlier that the cells of higher plants like those of various animals and insects did not have this power. Findings on the fixation of nitrogen by insects, not infrequently reported in scientific literature, were subsequently explained by the existence in the bodies of insects of symbiotic micro-organisms which fixed nitrogen. The symbiotic fixation of nitrogen was also established in the case of lichens to be due to the power of algae to fix nitrogen. It follows, therefore, that only lower plant organisms can assimilate atmospheric nitrogen.

With the power of blue-green algae to fix nitrogen firmly established, attention could be turned to the long-discussed question of the phylogenesis of the classical nitrogen-fixing bacterium, *Azotobacter*. There are a number of features in which *Azotobacter* resembles blue-green algae.

(1) The cell morphology and life cycle of *Azotobacter* have much in common with the morphological features which are typical of blue-green algae, the cells of which are round. This concerns not only the formation of a mucous capsule, the appearance of the resting cell, the power to multiply and so on but it also concerns minute details of cell structure. In both cases reticular structures corresponding to a central body can be demonstrated in the cells.

(2) The author gave some attention to the very low content of DNA in the cells of blue-green algae and *Azotobacter* (Imshenetskii, 1946). This was subsequently confirmed by the detailed investigations of Belozerskii *et al.* (1957), who

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found that *Azotobacter* contained 0·81–1·70 per cent DNA. We know that in the true bacteria (Eubacteriales) the DNA content is considerably higher and ranges between 2 and 6 per cent. Thus, in terms of the chemical composition of its cells, *Azotobacter* can be said to stand closer to blue-green algae than to bacteria.

(3) Certain ecological features also suggest such a relationship. Blue-green algae are not generally present in acid soils, and this is also characteristic of *Azotobacter*. The latter is also much more "hydrophil" than many other bacteria. Here, one should also note the very similar effect of certain micro-elements on the nitrogen-fixing power of *Azotobacter* and blue-green algae.

(4) Study of nitrogen fixation in blue-green algae and *Azotobacter* by the most modern methods has established a great similarity in the chemistry of this process.

There are, therefore, good grounds for regarding *Azotobacter* as an organism related to the blue-green algae. Whether it is a simplified alga which has lost its pigment and capacity for photosynthesis or whether it should be regarded as a form which developed earlier than the blue-green algae is still an open question. The former hypothesis would appear to be more correct but weightier judgements on this question will require detailed investigation of the biology of a number of other forms, and particularly of filamentous bacteria, which are very probably close to Cyanophyceae (Ponthothrix).

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EVOLUTION OF THE POWER TO FIX NITROGEN

Analysis of the origin and development of any function in lower plant organisms is always very difficult. It is rendered somewhat easier when the same physiological properties are seen in phylogenetically remote organisms. The view that the power to fix nitrogen developed simultaneously in primitive and much more complexly organized creatures is quite improbable even if only for the reason that these creatures developed at different times. A much more correct view is that the power to fix molecular nitrogen developed as a result of adaptation to definite ecological conditions in representatives of widely differing taxonomic groups in the course of a very long period of evolution. From this point of view the anaerobic *Clostridium pasteurianum*, or spore-bearing anaerobes closely related to it, should probably be regarded as the most primitive and apparently the earliest of all to develop as nitrogen-fixers. The existence of photosynthesizing bacteria which fixed nitrogen developed much later, and only after the appearance of oxygen was it possible for aerobic, nitrogen-fixing bacteria such as *Azotobacter* to develop. The nitrogen-fixing blue-green algae, which first lived only in waters but later went over to land and settled in the soil, evidently also appeared during this period.

The concluding stage in the evolution of nitrogen-fixing micro-organisms was the development of symbiotic relationships between nodule bacteria and leguminous plants. Theoretically, it can be postulated that, earlier, nodule bacteria had been free-living nitrogen-fixing forms in the soil, and that later, as a result of adaptation to the symbiotic form of life in the root system of the higher plant, they lost their power to bind nitrogen in pure culture.

When they turn to the past history of micro-organisms, microbiologists experience much greater difficulties than zoologists or botanists as, strictly speaking, palaeomicrobiology as a science does not exist. Despite the exceptional importance of imprints of individual micro-organisms, mainly filamentous bacteria, microbiology cannot make use of these finds for the development of a phylogenetic system, and still less for the explanation of the evolution of any function. Utilization of the information on the state of the earth's atmosphere and on abiogenic evolution of organic substances on this planet, which Oparin (1957) has expounded in such detail in his widely known book, may prove much more promising.

Analysis of the evolution of physiological features in micro-organisms is impossible without examination of the conditions of their existence, and our wealth of information on the nature of the medium in which, at some time or other, micro-organisms lived helps us to reconstruct the possible course of their evolution. This palaeo-ecological approach began to be used in relation to micro-organisms only comparatively recently and it deserves very serious attention. In this connexion it can be postulated that forms assimilating ammonia nitrogen predominated among micro-organisms during the period when the atmosphere of the earth contained considerable quantities of ammonia. Subsequently, the ammonia content fell rapidly and disappeared almost completely from the atmosphere which continued to contain gaseous nitrogen. During this period there could have been gradual adaptation to the assimilation of atmospheric nitrogen among organisms which found themselves in conditions of nitrogen starvation. Confirmation of this hypothesis is afforded by the fact that, in the case of the nitrogen-fixing micro-organisms of today, a switch from gaseous nitrogen to ammonia nitrogen takes place without any period of cell adaptation.

The question of sources of energy for primitive nitrogen-fixers is of considerable interest. An important factor for the solution of this problem would be the finding of nitrogen-fixing bacteria which used various hydrocarbons as energy material. And even though such nitrogen-fixing bacteria have not been found, two facts should be noted in this connexion. First, we know that, when hydrocarbons are broken down by heterotrophic bacteria, there is formation of intermediate products and particularly of various organic acids which can serve as an excellent source of energy for nitrogen-fixing micro-organisms. Secondly, it should be emphasized that a number of chemical compounds closely related to hydrocarbons are used by nitrogen-fixing bacteria much better than carbohydrates as energy material. This applies particularly to *Azotobacter* which grows on media containing benzoic and hydroxybenzoic acids.

The power to utilize monosaccharides and the products of polysaccharide hydrolysis apparently developed later in nitrogen-fixers when, as a result of photosynthesis, carbohydrates began to accumulate in the cell of higher plants.

With the development on the earth of photosynthesizing bacteria, the appearance among them of special forms having the power to fix atmospheric nitrogen without at the same time using organic compounds as a source of energy became a possibility. In equal degree this applies also to nitrogen-fixing blue-green algae

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which, like the nitrogen-fixing photosynthesizing bacteria, are classed as the most "perfect" autotrophs.

In speaking of the development and evolution of a process for linkage of atmospheric nitrogen, one must first of all dwell on the distribution of the hydrogenase system among micro-organisms. On the assumption accepted by us earlier that anaerobic micro-organisms were present on the earth much earlier than aerobic forms, hydrogenase must be regarded as one of the most "ancient" enzymes, as it is typical of anaerobes. One may not agree that the hydrogenase system is possibly the equivalent of cytochromeoxidase, which is absent in anaerobic bacteria, but there is no doubt that the activity of the hydrogenase system has a great effect on the spread of electrons in anaerobic forms. Much new information has been obtained in investigations on the hydrogenase systems of various anaerobic species of *Clostridium*, which we regard as the original nitrogen-fixing bacteria. A powerful hydrogenase system was subsequently discovered in the classical aerobic nitrogen-fixer, *Azotobacter*, and it was also found that this system was active only when *Azotobacter* fixed atmospheric nitrogen. Immediately *Azotobacter* was placed in a nutrient medium containing a salt of ammonia, the activity of the hydrogenase system climbed sharply. These observations suggested that hydrogenase participated in the fixation of nitrogen. Such a connexion is also indicated by the results of the numerous investigations of Wilson *et al.*, who studied the hydrogenase and nitrogenase activities of acellular preparations obtained by the destruction of *Azotobacter* cells by various methods (Hyndman *et al.*, 1953; Wilson & Burris, 1953). It is highly probable that hydrogenase systems have undergone change in the course of evolution. The fact that carbohydrate does not suppress nitrogen-fixation in anaerobic nitrogen-fixing bacteria and photosynthesizing bacteria confirms this. On the other hand, carbohydrate has an inhibiting effect on the fixation of nitrogen by aerobic nitrogen-fixing bacteria. Yet the mere fact that the hydrogenase systems of aerobic bacteria function in the presence of oxygen is indicative of great change in this system, of its resistance to the new aerobic conditions of life of the micro-organisms. Nor is it possible to agree with the opinion expressed by Oda (1957) that, as a result of evolution, the hydrogenase system has been combined with enzyme systems connected with certain fractions of the cell. This view is supported by the following fact. Over a considerable period of time the author of this paper tried unsuccessfully to reproduce acellular fixation of nitrogen with preparations obtained by filtration of a suspension of destroyed *Azotobacter* cells (Imshenetskii *et al.*, 1956). It was subsequently demonstrated by other investigators that acellular fixation of nitrogen was effected, not by filtrate, but by the particles which remained after destruction of the cells of nitrogen-fixing bacteria. It was established that the dehydrogenase and nitrogenase systems were connected with particles still present after disintegration of the cells.

The study of hydrogenase activity in micro-organisms has increased the list of nitrogen-fixing bacteria considerably. The depressing effect of nitrogen and ammonia on the excretion of hydrogen by photosynthesizing bacteria has led us to

suspect that a nitrogen-fixing system could be demonstrated in these organisms. These investigations afforded further confirmation that hydrogen metabolism and nitrogen metabolism are closely linked in nitrogen-fixing micro-organisms.

It can, therefore, be regarded as proved that hydrogenase activity in nitrogen-fixing micro-organisms is inseparably connected with the power to fix molecular nitrogen. Despite a considerable number of investigations on the subject, there is still no generally accepted conception of the actual mechanisms responsible for nitrogen fixation, and one can but agree with Pochon & de Barjac (1960) and Thornton (1954), who arrived at the same conclusion as a result of an analysis of the present status of the problem. One can speak of the existence of several theories and the results of certain experimental investigations can be adduced in support of each of them. Experiments with ^{15}N afforded convincing proof of the formation of ammonia as a product formed at the end of fixation and the commencement of the period of assimilation of the fixed nitrogen, a product associated with its attachment to the "organic carbon skeleton". This mode of fixation has been established for various nitrogen-fixers. It has been established by the use of ^{15}N that, irrespective of whether the micro-organisms assimilate gaseous nitrogen or ammonia, a considerable fraction of the labelled nitrogen is found in the glutamic acid formed as a result of restorative amination of α-oxo-glutaric acid. When there is insufficient carbonic acid in these cells there is discharge of glutamine, asparagine and ammonia from the cells into the medium.

A particularly high content of labelled nitrogen was observed in the ammonia and this was also to some extent confirmatory of the role of ammonia as an intermediate product in the fixation of nitrogen.

On the other hand, Virtanen attaches great importance in the chemistry of nitrogen fixation to hydroxylamine, which combines with oxaloacetic acid, later reduced to aspartic acid (Virtanen *et al.*, 1936; Virtanen & Jarvinen, 1951). The objections to the participation of hydroxylamine in the process of nitrogen fixation amount to the fact that nitrogen-fixing micro-organisms do not grow in media containing hydroxylamine because of its great toxicity. The fact that this toxic effect of hydroxylamine has been observed refutes the suggestion that it does not penetrate the bacterial membrane. In the same way nitrogen-fixers do not assimilate hyponitrite contained in the medium, although this is regarded as one of the possible primary products of nitrogen fixation. The latter hypothesis and a number of others have arisen as the result of attempts to find similar reactions in the process of nitrogen fixation and that of nitrate reduction.

Underlining the difficulties in respect of energy arising in attempts to prove activation of nitrogen in nitrogen fixation, Fedorov (1952) put forward a theory that the cells of nitrogen-fixing micro-organisms contain a special enzyme containing CO, NH_2 and COOH groups. The carboxyl group, reacting with N_2, forms the CO—NH_2 bond. The hydrazine derivatives formed enter into the composition of the bacterial protein with the formation of NH_2OH and NH_3 as by-products.

It is not part of our purpose here to explain the existing theories of the chemistry of nitrogen fixation. We have instanced some of the views solely for the purpose of

emphasizing that, despite undoubted advances in this field, we still do not have any generally recognized conception and this makes it difficult to study the process of nitrogen fixation in its comparative aspects. Even now, however, it is possible, although at variance with widely prevailing traditional views, to underline the similarity, if not the unity, that exists in the chemistry of nitrogen fixation in different organisms and to note several features of nitrogen fixation in the different micro-organisms.

It is very probable that there is direct reduction of nitrogen to ammonia in anaerobic bacteria, the earliest nitrogen-fixing bacteria to appear. No formation of intermediate oxidation compounds was observed here. A characteristic feature of *Clostridium*, a typical representative of this group of nitrogen-fixing bacteria, is the absence of any depression of nitrogen fixation in the presence of H_2 and CO.

It is very probable that, with the development of aerobiosis, the chemistry of nitrogen fixation became somewhat different. In the aerobic nitrogen fixation produced by *Azotobacter* or nodule bacteria there is, initially, formation of oxidation compounds, and particularly hydroxylamine, which is later converted to NH_3. In the opinion of Virtanen, the formation and excretion of nitrogen oximes during fixation confirms that the process follows just such a course. Subsequently, the oximes may be reduced with the formation of, more particularly, aspartic acid. The development of amino acids, and primarily glutamic, aspartic or alanine, is observed in a wide variety of nitrogen-fixing bacteria, both anaerobic and aerobic, and there are good grounds for regarding amino acids as intermediate products in nitrogen fixation. The formation of amino acids is easily explained by reactions between hydroxylamine and various keto acids, and the possibility of such reactions in the process of nitrogen fixation is admitted by many.

With the appearance of higher plants on the earth, symbiosis between nodule bacteria and leguminous plants became possible. The conditions attaching to the symbiotic fixation of nitrogen were quite different from those in which nitrogen was fixed by free-living micro-organisms. The development, multiplication and nutrition of the nodule bacteria, as well as the fixation of nitrogen, occurred in the tissues of higher plants and this could not but produce material changes in the biology of both the higher plants and the bacteria. We dwell briefly on certain features of nitrogen fixation as effected by nodule bacteria.

(1) A red haemoglobin pigment, the haemin group of which is quite similar to the haemin group of blood haemoglobin, can be readily demonstrated in the central parts of the nodules, that is in the area where nodule bacteria fix nitrogen most actively. The haemoglobin contained in the nodules develops only as a result of symbiosis, as neither leguminous plants nor nodule bacteria separately form haemoglobin. The haemoglobin can only be demonstrated in the nodules during a period of intense nitrogen fixation by the nodule bacteria. The quantity of nitrogen fixed is directly proportional to the quantity of haemoglobin contained. What the function of the haemoglobin is in nitrogen fixation is still not clear, although it may possibly take part in oxidation processes or, because of the ion it contains, function as a catalyst. The essential point from the standpoint of comparative

physiology is that the conditions of symbiosis led to the formation of haemoglobin, which is absent in free nitrogen-fixing bacteria, and was directly related to the intensity of nitrogen fixation.

(2) It is well known that free-living nitrogen-fixing bacteria fix nitrogen most actively in young cultures containing multiplying cells. On the other hand, in the case of nodule bacteria the most active period in respect of nitrogen fixation coincides with the formation of bacteroids in the nodules. These bacteroids can, with great justification, be classed as "resting" or "reactive" forms as most of them, when transferred to fresh medium, do not multiply. Thus, as far as symbiotic fixation is concerned, the biochemically active bacterial cells are only capable of manifesting their vital activity when in the cells of higher plants.

(3) Adaptation to the conditions for life created within the plant cell has led to the position that special conditions, which are difficult to reproduce in the laboratory, have become necessary for the fixation of nitrogen. Only this would explain the fact that at the present time there are no unassailable proofs that nodule bacteria are capable of fixing atmospheric nitrogen in pure culture under laboratory conditions.

(4) Amino acids are formed in the nodules during the process of nitrogen fixation in such large quantities that they are easily demonstrated in the soil around the nodules. It is highly probable that the amino acids reaching the soil can be assimilated by the leguminous plants. If this is so, this entire process of the formation and excretion of amino acids can be regarded as one of the adaptations of higher plants, leading to the steady entry into the soil of additional sources of nitrogenous food.

The fixation of atmospheric nitrogen by micro-organisms has attracted the attention of representatives of various branches of science. This process returns several times more nitrogen to the soil annually than the quantity introduced in organic and mineral fertilizers; the interest of agronomists and agricultural chemists therein is, therefore, understandable. Research on the chemistry of the biological fixation of nitrogen is also of exceptional importance as it undoubtedly brings us nearer to the remote but nevertheless attainable end, namely, the reproduction under laboratory conditions of the catalytic process for the fixation of atmospheric nitrogen, a reproduction based on the findings of comparative biochemistry on nitrogen fixation.

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ALTERNATIVE PATHWAYS OF CARBOHYDRATE METABOLISM IN RELATION TO EVOLUTIONARY DEVELOPMENT

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There now appears to be general acceptance of the view that the earliest forms of life arose in an environment which already contained most of the compounds which we recognize as components of the living cell. The experiments of Miller (1955), Butlerov (1861), Fischer & Tagel (1889) and many others have suggested that carbohydrates, in addition to amino acids and purines, may have been formed under primitive Earth conditions. The early atmosphere was a highly reducing one, and since the formation of oxygen through photosynthesis appears to be a relatively recent development, it is probable that the first utilization of carbohydrate was by means of a fermentation mechanism. Respiration, the utilization of oxygen for energy production, appears to have developed later as a modification of pre-existing fermentation mechanisms.

The pathways of carbohydrate metabolism are designed to satisfy three major requirements of the cell. These are (1) for energy, (2) for precursors or building-blocks for synthesis and (3) for reduction or oxidation mechanisms for converting these precursors to the appropriate intermediates or end-products for incorporation into cellular components. If we accept the principle that the first living organisms arose in a very complex medium and possessed limited synthetic capacity, then their primary, perhaps sole, requirement was for a source of energy to drive the endergonic assembly mechanisms. It is assumed that the environment provided preformed precursors in the appropriate form.

Our problem is therefore to examine the known pathways of carbohydrate metabolism and to evaluate them in terms of their role in meeting the new requirements which arose as the organic components of the environment were depleted, one by one.

THE PYRIDINE NUCLEOTIDES

When Warburg *et al.* (1935) discovered triphosphopyridine nucleotide they named this the "wasserstoffübertragendes co-ferment", in contrast to cozymase which acted as the coenzyme of fermentation. The implication was that TPN was the coenzyme associated with the respiratory activity of the cell, while DPN was associated with electron transfer mechanisms in fermentation. In the light of what has been learned during the past 25 years, it is necessary to modify this concept only slightly to preserve its original significance. Thus, the requirement for a second coenzyme, TPN, is a consequence of the aerobic metabolism of the cell, even though TPN itself may not be directly involved in the transfer of electrons to oxygen. In this way, by utilizing two coenzymes rather than one, the cell is able to establish a rapid transfer of electrons to oxygen and at the same time maintain a coenzyme in the reduced form to provide electrons for the synthesis of highly reduced components of the cell. This can be summed up, as Kaplan *et al.* (1956) have

done, in Fig. 1, where DPN is shown in the path of electrons to oxygen, while TPN acts as an intermediate between one substrate and another.

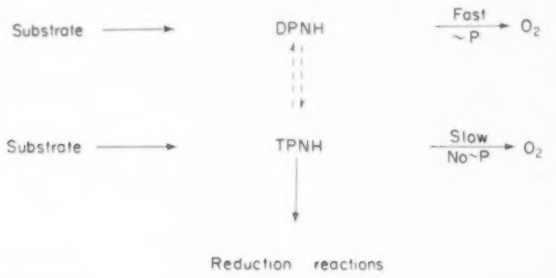

FIG. 1. Pyridine nucleotides in electron transport. From Kaplan *et al.*

According to this hypothesis, the first coenzyme from an evolutionary point of view must have been DPN. When oxygen appeared in the atmosphere and the electron transport mechanism from DPNH to oxygen developed, the necessity arose for a second coenzyme, which could carry on some of the old reducing functions of DPNH which were no longer possible in view of very effective removal of reduced coenzyme by oxygen. The same result would be accomplished, and evidently was, by sequestering the coenzyme in different parts of the cell so that it would be oxidized only in those regions containing the oxidases. This, however, is a more complex solution and must, therefore, have been a much later evolutionary development.

THE EMBDEN–MEYERHOF PATHWAY

Because of its widespread occurrence in micro-organisms, plant and animal cells, there can be little question about the primitive nature of the Embden–Meyerhof pathway. This mechanism for the dissimilation of carbohydrates must have arisen very early, since in most higher organisms it forms the basis for aerobic metabolism via the tricarboxylic acid cycle. It is remarkable how similar the individual reactions of the Embden–Meyerhof pathway have remained in cells of diverse living forms, such as yeast, animal or plant cells. The pathway can hardly be considered to have arisen independently in these forms and must have originated in a common ancestor. Small differences do exist, as, for example, in the aldolases of yeast and muscle, the former showing a metal requirement which is lacking with the latter (Warburg & Christian, 1943). Yet in other instances the similarities are so great as to have led Velick & Undenfried (1953) to compare yeast and muscle glyceraldehyde 3-phosphate dehydrogenases with respect to their amino acid composition. Only in the last steps of the Embden–Meyerhof pathway do we find divergences; in a relatively few types of cells (plants, fungi, some bacteria) ethanol and CO_2 are the end-products. Animal and most bacterial cells produce lactic acid and still others more complex fermentation products, such as

acetyl methyl carbinol, acetone and butyric acid. On the basis of its phylogenic distribution alcoholic fermentation seems to be a later development arising in cells which have already developed the capacity to form other fermentation products from pyruvic acid.

THE HEXOSEMONOPHOSPHATE OXIDATION PATHWAY

Although nucleic acids must be present for life as we know it today, we know little of the early origin of these complex polymers. The question is closely intertwined with that of energy metabolism, since similar nucleotides are involved in the latter and in the synthesis of the polymers. Our present discussion will be limited, however, to the problem of the pentoses which are universal components of nucleotides and nucleic acids. Whether these were preformed in the original hydrosphere, or appeared later and replaced other more primitive types of genetic material, we do not know, but it is of interest to consider the development of present pathways.

All cells which have been examined thus far possess at least one mechanism for the formation of pentose from hexose, and in most cells more than one may be detected. Two of these pathways for the formation of pentose, when taken together, constitute a cyclic process (Fig. 2) although it is unlikely that the cycle has any

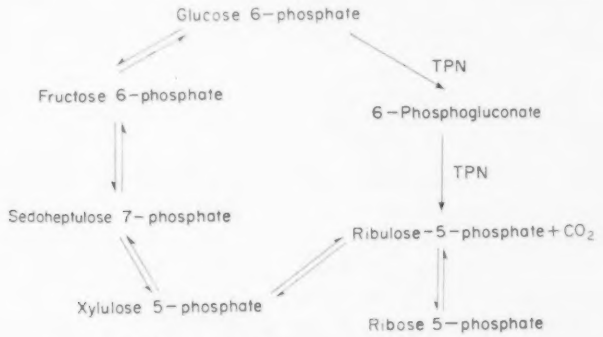

FIG. 2. Mechanisms of pentose phosphate formation.

general significance as such. Experiments with labeled hexose have established that in most cells the direction of flow in each pathway is from hexose phosphate to pentose phosphate, rendering a cyclic mechanism exceedingly unlikely (Horecker & Mehler, 1955).

The so-called non-oxidative pathway, involving transketolase and transaldolase, may be considered to be the more primitive for several reasons. In the first place, these enzymes appear to be universal in their distribution. They have been detected in all cells which have been adequately studied. This is in contrast to the dehydrogenases which, as we shall see, appear to be lacking in many organisms, particularly micro-organisms. In the second place, these same reactions which provide the pentose phosphate for nucleic acid synthesis are also incorporated into

the photosynthesis and chemosynthesis mechanisms of plants and micro-organisms, They must therefore have existed when these processes developed. And, finally, the dehydrogenase reactions appear to satisfy the need for an intracellular reducing mechanism, a need which would not have existed so long as the hydrosphere itself provided a reducing environment.

What may be a primitive form of the hexose monophosphate oxidation pathway is to be found in certain micro-organisms. *Leuconostoc mesenteroides*, for example, possesses a fermentation mechanism (Gunsalus & Gibbs, 1952; DeMoss, 1953, 1954) which differs from the Embden–Meyerhof pathway in that the oxidizing steps are at the level of glucose 6-phosphate, rather than triose phosphate as in ordinary lactic or ethanol fermentation (Fig. 3). These reactions involve DPN,

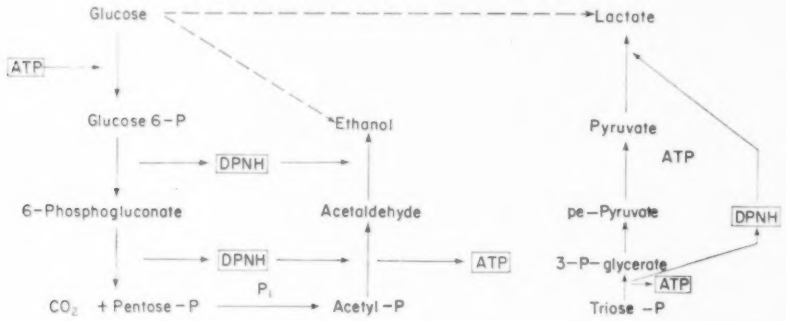

FIG. 3. Glucose fermentation in *Leuconostoc*.

rather than TPN, which is a further testament to their early origin. It is rare that we find glucose 6-phosphate and 6-phosphogluconate oxidation linked to DPN rather than TPN. In accord with the hypothesis presented earlier, an organism which lives by fermentation, without the powerful DPNH oxidases which characterize respiring cells, has no need for a second coenzyme to maintain a reducing potential. The same coenzyme which acts in the fermentation process can be used for reduction. It is noteworthy that Cynkin & Gibbs (1957) were unable to detect the reduction of TPN by glucose 6-phosphate in *Clostridium perfringens*.

$$\text{Glucose} \xrightarrow{\text{TPNH}} \text{Sorbitol} \xrightarrow{\text{DPN}} \text{Fructose}$$

FIG. 4. Conversion of glucose to fructose in the seminal vesicle (Hers, 1960).

Another aspect of the fermentation mechanism of *Leuconostoc* is of interest in connexion with possible early pathways of glycolysis (Hurwitz, 1958). In this organism, acetyl phosphate formed in the phosphorolytic cleavage of xylulose 5-phosphate (Fig. 3) is reduced to acetaldehyde and finally to ethanol, employing the same type of mechanism found by Burton & Stadtman (1953) in *Clostridia*.

This may represent a primitive type of reducing mechanism in fermentation. It will be recalled that Harting & Velick (1954) in the Coris' laboratory found acetyl phosphate to be reduced by triose phosphate dehydrogenase. It may well be that this is a vestige of a primitive enzyme system which developed originally for the reduction of acetyl phosphate, but which became modified for the more efficient reactions involving triose phosphate. Although phosphoketolase-type reactions are lacking in most cells, their occurrence in species of *Leuconostoc*, *Lactobacillus* (Heath *et al.*, 1958) and *Acetobacter* (Schramm & Racker, 1957) suggests that they may have been involved in primitive fermentation mechanisms.

In summary, we may consider that the oxidation of glucose 6-phosphate by DPN was a very early development, perhaps arising alongside of the Embden–Meyerhof pathway as an alternate or additional fermentation mechanism. With the appearance of oxygen in the atmosphere and the consequent depletion of the reducing substance of the hydrosphere, the need arose to provide mechanisms for the formation of reduced cytoplasmic components and at the same time to provide a source of energy less wasteful of the dwindling supply of organic materials. This led to the appearance of respiratory mechanisms, which derived energy from oxidation of DPNH. A new coenzyme was needed, which would not be so rapidly oxidized, with which to preserve a reducing potential. This need was filled by TPN, which Glock & McLean (1955), Jackson & Kaplan (1957) and others have shown to occur in cells mainly in the reduced form, unlike DPN, which is almost fully oxidized. Through TPN the energy supplied by the oxidation of glucose 6-phosphate is channeled into endergonic reduction reactions. A glance at any metabolic map shows us that reactions which proceed in the direction of reduction usually involve TPNH, rather than DPNH. Glucose 6-phosphate and 6-phosphogluconate have become substrates for TPNH production and incidentally precursors of pentose phosphate.

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INTERCONVERSION OF SUGARS WITHOUT PHOSPHORYLATION

It is difficult to tell when phosphorylated intermediates assumed their important role in metabolism. Many species of organisms derive energy from the direct, but limited, oxidation of sugars and polyhydric alcohols, as in the oxidation of glucose to gluconate, and the oxidation of glycerol to dehydroxyacetone. These may well be vestiges of mechanisms which utilized oxygen through primitive flavin and cytochrome systems.

Recently a pathway of carbohydrate metabolism has come to light which is to be found in such diverse forms as the filamentous fungi and the mammalian foetus and seminal vesicle. If we accept the traditional view that ontogeny recapitulates phylogeny, then this must indeed be a most primitive pathway.

In the seminal vesicle, as Hers (1960) has shown, glucose is converted to fructose by the mechanisms shown in Fig. 4. The sequence involves the reduction of glucose to sorbitol, with TPNH as the coenzyme and oxidation of sorbitol to fructose by DPN. In the placenta of some species, including the sheep and cow, glucose is thus converted to sorbitol which is carried to the liver of the foetus

where it is in turn converted to fructose by the DPN-linked enzyme. The physiologic function of this mechanism remains obscure. It is of interest, however, that the enzyme found in the seminal vesicle is reactive with a large number of aldoses, including a number of hexoses as well as pentoses and trioses. The same is true for the enzyme found by Chiang & Knight (1960a, b) in *Penicillium chrysogenum* and other molds and by Veiga *et al.* (1960) in species of *Candida*. In *Candida*, for example, D-xylose is converted to D-xylulose by way of xylitol. This organism also contains a very active glucose 6-phosphate dehydrogenase, suggesting that glucose metabolism involves the pathways shown in Fig. 5. The same enzyme which converts glucose to sorbitol will form xylitol from xylose; the absence of specificity is reminiscent of the enzyme found in the seminal vesicle.

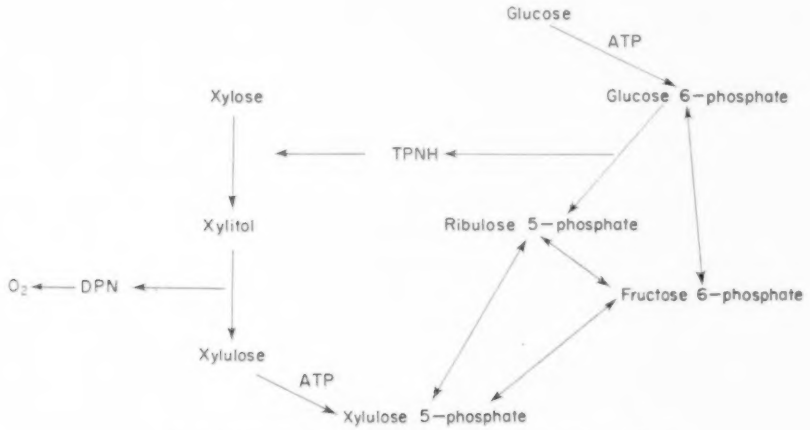

FIG. 5. Glucose and pentose metabolism in *Candida*.

While direct proof is yet lacking, the occurrence of this enzyme in germinal and foetal tissue and its lack of a specialized function, together with its similarity to the enzyme found in a primitive filamentous micro-organism, suggest a common origin early in the development of carbohydrate metabolism.

CONCLUSION

It is, of course, of tremendous interest to speculate on the origin of life and the evolutionary development of the intricate pathways of metabolism which characterize modern forms. Such speculation is useful only when it is based on a thorough understanding of the nature of these pathways and their function in contemporary forms. As Oparin (1957) has pointed out, "a detailed study of the processes of metabolism is the thing which can lead towards a solution of the problem of reproducing it [life] artificially". We have come a long way in the last 30 years; the solution to the problem no longer appears to be beyond our grasp.

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ORIGIN AND EVOLUTION OF METABOLISM

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THE doctrine that, in the process of the development of life, there was successive formation of metabolism and the catalytic and structural cell mechanisms which are responsible for it, is receiving ever-increasing attention from biochemists. There is no doubt, however, that the starting point for the building of this branch of evolutionary biochemistry is the formation of conceptions on the earliest simplest forms of metabolism, already being built up even when life was in the process of emerging and in the very early stages of its development. This difficult task is in fact the subject of this paper. It cannot be accomplished by a simple study of metabolism in living organisms, even though the most primitive in this respect are studied. It at once becomes more and more evident that their metabolism is itself the product of a very long evolutionary development. It is conceivable that the whole of the first half of the time that life has existed on earth (i.e. somewhere about a thousand million years or more) was spent on the perfecting of metabolism and cell structure, on conversion of the initial systems for the engendering of life into the extremely primitive ancient forms of bacteria and algae.

Only careful comparison of the organization of metabolism in a wide range of representatives of the entire existing animal kingdom will enable us to distinguish certain links in metabolism, certain combinations of biochemical reactions, which are strikingly universal, common to the entire animal kingdom. This will point to the extreme antiquity and will at the same time serve as a guiding thread for the creation of a probable schema of the original appearance and initial evolution of metabolism. The compiling of such a schema will be useful in that it will serve as a working hypothesis for the creation of model experiments reproducing the individual stages through which metabolism passed during its formation under natural conditions.

It is now the generally accepted opinion that various organic substances could have been formed on our planet even before the appearance of life by purely abiogenic means in the waters of the then existing seas and oceans. Thus, these waters were at some time or other converted, as it were, into a peculiar form of nutrient broth, the total concentration of which was fairly considerable (according to the calculations of Urey it reached an average of several per cent).

The chemical conversions, formation and breakdown of organic substances that occurred there were, however, radically different in type from those peculiar to living organisms. In the latter, as a result of the organization which has already taken place in them, a sequence of individual reactions is strictly co-ordinated into a single metabolic network. Consequently the sequence of processes occurring

here is quite purposeful and is capable of leading to constantly repeating synthesis sometimes of quite complex and specific compounds, which can thus be rapidly built up in considerable quantities in the cell.

Such a sequence was, of course, completely absent in the original nutritive broth. Just as in simple aqueous solutions of organic substances, the chemical conversions occurring in the broth merely followed the general laws of physics and chemistry. They had no directed, organized character and occurred in all directions over the widest field of chemical possibilities.

Here, any substance could undergo change in a great variety of ways and individual reactions intercrossed in the most curious manner. In this medium, therefore, it was possible for a great variety of all the possible organic compounds and their polymers to develop, but the more complex and specialized a particular substance was, the greater the number of reactions which had to participate in its formation would be, the less likely its formation became and, consequently, the lower would be the possible concentration of this specific substance in the original broth. It is, therefore, easy to imagine the possibility of wide abiogenic formation of sugars, amino acids, purine and pyrimidine bases, as well as their non-specific polymers, but it is extremely improbable that there was abiogenic formation in this medium of proteins or nucleic acids, which are formed by specific, strictly determined arrangements of amino acid or mononucleotide radicals.

Despite the complex obviousness of the position here, the current opinion in scientific literature is still that all the chemical ingredients of protoplasm characteristic of the modern cell, such as enzymes, nucleic acids and other complex compounds, the intramolecular structure of which is very well or indeed perfectly adapted to the fulfilment of certain definite biological functions, must have arisen in the nutritive broth. And, it would seem, the appearance of organisms occurred as a result of the combination of ingredients into a single system, in much the same way as a machine is built from previously prepared parts.

Yet, study of the biosynthesis of enzymes proves altogether convincingly that the formation of any enzyme molecule requires a whole series of precursor enzymes with their joint actions combined into a single system. Given the presence of such a system, one can imagine the synthesis of enzyme proteins even apart from cells, even in model experiments, but this is completely impossible, inconceivable, merely in a solution of organic compounds, in the absence of a complexly organized system of catalytic and structural mechanisms.

The same can be said of the synthesis of specifically constructed nucleic acids. This also requires for its realization the co-ordinated action of a number of enzyme systems and other biological mechanisms. More particularly also, virus nucleoprotein is incapable of multiplying either in any broth or other solution, or in fact in any simple medium, no matter how many are tested in this respect. Increase in the quantity of virus, its synthesis, occurs only as a result of the metabolism of the host cells. Numerous interacting enzymes and other systems participate in this synthesis. The advances of recent years have led us to hope that these systems will be successfully isolated some day and that it will be possible to synthesize virus in

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model experiments by their organized interaction, but models constructed in this way will not, of course, have anything in common with the simple aqueous solution of the organic substances of the original broth. So that even if, by some absolutely exceptional chance, a single molecule of virus nucleoprotein did appear (which is in itself extremely improbable), this would not have any material effect on the further course of events as this molecule would be unable to multiply in the original broth.

A characteristic feature of life is that it is not simply scattered in space but is represented by individual, very complicated systems, delimited from the external world—by organisms. The emergence of these can only occur after prolonged evolution, improving on some very much simpler initial systems, derived from the original simple solution, the nutritive broth. These systems must have been very close in chemical composition to the composition of the medium surrounding them, not because the latter was exceptionally complex and specialized but, on the contrary, because the composition and structure of the systems themselves were incomparably simpler than anything that we now observe in the living nature surrounding us.

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Originally, such systems must have been simply separated parts of the original broth, isolated from the general solution by some surface membranes or films, but capable of interacting with the medium surrounding them. The development of such systems would not represent anything special or exceptional. It could occur quite often under ordinary conditions and in a great variety of ways. For example, Goldacre has recently described the formation under natural conditions of small closed bladders enclosed in a lipoprotein membrane. These bladders develop from folds produced by wind in the films seen on the surface of certain natural waters.

When high molecular substances were present in the solution (even in very low concentrations of the order of 0·001 per cent), the formation of coacervate droplets occurred readily in them. There was, at the same time, considerable concentration of the substances in the droplet, and the latter was separated from the surrounding solution by a clearly defined surface. Bungenberg de Jong has shown that, in the presence of lipids, the surface of coacervate droplets can also assume a protein–liquid membrane, constructed after the manner of a sandwich. It is important to note that membranes of this type constitute a structural element which is absolutely common to all contemporary living bodies without exception. This points to their very early development in the process of the evolution of life.

The interaction between the coacervate droplet (or other analogous system) and the surrounding medium was expressed primarily by the power of the droplet to absorb substances selectively from the surrounding solution. If, however, the interaction were limited to this, equilibrium would develop rapidly and this would exclude any further evolution of the original system. For this to occur it was necessary that chemical processes of some kind should take place in the system at the expense of the organic substances reaching it from the external medium. In this case the droplet acquired the properties of an open system and can be represented by the elementary diagram (Fig. 1), in which the dotted line indicates the

boundary of the system or the film covering it, S and Z represent the external medium and A and B, the substances participating in the reaction.

The substance A, continuously entering the system from the external medium, is converted with the liberation of a certain quantity of energy into substance B, which then diffuses into the external medium. The result is that static equilibrium between the system and medium cannot be attained and a stationary state is created, with a constant and one-way flow of substance through the system, dF

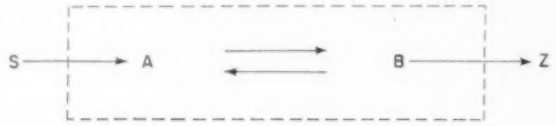

FIG. 1.

remaining constant all the time on account of the energy coming with the substance A from the external medium.

Open systems of this kind and the kinetics and thermodynamics of the reactions occurring in them were recently studied by Passynskii and Blokhina in micromodels in which the surface film was represented by a cellophane membrane. But similar phenomena can also be observed in coacervate droplets if some chemical reaction is made to occur in them.

As for the organic substances in the original broth which served as the main source of energy for the original open system, one can imagine an infinite number of processes leading to the liberation of free energy applicable to these. But, in the conditions of a reduction hydrosphere, the most effective of these were undoubtedly redox reactions, processes connected with transfer of hydrogen (of an electron).

Even on the evidence of these purely chemical considerations, it becomes very probable that reactions of this type were the earliest links in the creation of metabolism. This is also confirmed by comparative biochemical findings, as it is impossible to point to any other more universal link in the metabolism of all organisms without exception. Kluyver correctly considers the presence in parts of the cell of continuous and directed movement of electrons as the most essential sign of the living state.

Oxidation–reduction reactions, and particularly the reactions for transfer of hydrogen, may be accompanied by splitting of the molecules or by their condensation. In the former case the concentration of the substances entering the system is constantly being reduced and this creates conditions for their constant fresh arrival from the external medium. When the opposite is the case, the concentration of the products of the reaction is constantly increasing and this determines their diffusion into the external medium of their accumulation within the system.

Constant movement of this nature through the system can only occur, however, when the speed with which the reaction within the system is completed exceeds the speed of the same reaction in the external medium. This requires the presence in the system of some catalyst capable, even though in small degree, of accelerating

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this reaction. This catalyst need not necessarily arise in the system itself; it may enter from the external medium, but in this case it must be concentrated or activated in the system. A phenomenon of this nature can be demonstrated in a number of model experiments. Hence it becomes absolutely clear that the participation of catalysts (even though very primitive) was essential in the very early stages of the development of the original system.

Theoretically, it is possible to think of a colossal number of different atomic groupings, radicals, organic or inorganic compounds which could serve as catalysts for the hydrogen (electron) transfer reactions. And it is conceivable that a great variety of substances from the external medium were utilized as catalysts in the first stages of the evolution of the different original systems.

The exceptionally important point was, however, that out of all these boundless possibilities, living nature, still in the very early stage of its development, selected only a minimum number of compounds which even today are the universal carriers of hydrogen, not only for absolutely all representatives of the animal world but also in the most varied system of exchange. These were, first of all, pyridine and later flavine phosphonucleotides. We meet with pyridine nucleotide as one of the most important biochemical mechanisms both in bacteria and in higher plants and animals, both in heterotrophes and autotrophes, both organisms accumulating and oxidizing various sugars and animals whose source of carbohydrate food are phenols and other related hydrocarbon derivatives. Possibly the original systems first developing had compounds of simpler structure as catalysts for the redox reactions occurring in them, but there is no doubt that pyridine nucleotide was used as a carrier of hydrogen even in those remote times when the tree of life was still unbranched, or, at any rate, that part of it which was the starting point for the formation of the entire contemporary animal world.

We have already pointed out that the products of the reaction effected in an open system can diffuse into the external medium, but they can also be retained in the system (particularly in the form of polymers), and this leads to increase in the mass of the system, to its growth. This was demonstrated in a model experiment by Serebrovskaia and her co-workers. In this experiment synthesis of polynucleotide occurred in coacervate droplets at the expense of the diphosphomononucleotides of the surrounding medium. And the mass of the droplet increased. A phenomenon of this nature, entirely based on a single reaction, can, however, occur only in exceptional cases, when the chemical compounds utilized from the external medium are very rich in energy.

The progressive evolution of the original systems required that compounds of this nature should be formed in the system itself. This, however, required that the energy developed in the process of the redox reaction should not be dissipated simply in the form of heat, but should be used for the building of compounds with an increased store of energy.

The organization of living material is fundamentally based on mechanisms by means of which free energy from chemical reactions becomes available for various syntheses and other processes requiring energy. What was very important,

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therefore, was not so much that energy should be liberated in the original system as that conditions should be created for its utilization. But one individual reaction was still completely insufficient for this and there was need for its conjunction with other reactions for the development of special "coupling mechanisms".

Thus, at this higher stage in the evolutionary development of the original systems not one but several co-ordinated reactions, leading to the production of compounds with increased energy content, had to be effected in these systems.

The combinations of reactions most universal for all living nature (although not by any means the most perfect) are those which are effected anaerobically and in which pyridine nucleotide takes part. The high-energy compound then formed is in most cases adenosine triphosphate which can be demonstrated in all contemporary organisms without exception and which is a quite universal source of energy for the most varied vital phenomena, and particularly for a number of biosyntheses. An important part in these syntheses is played by yet a third (along with DPN and ATP) and likewise biologically ubiquitous catalyst, namely co-enzyme A.

So at this stage in the development of individual systems, metabolic networks characteristic for each system were formed composed of a still comparatively small number of reactions catalysed by a few co-enzymes. These networks of reactions were gradually improved in the process of evolution by natural selection of individual systems.

Yet, on the thermodynamic considerations put forward by Prigogine and the recent investigations of Mizunoja, the progressive evolution of metabolism must necessarily have proceeded towards increasing complexity of the courses of reactions and increase in the number of reaction links in the chain of metabolism. Consequently, metabolic links which existed earlier were successively amplified by more and more new reactions and we now observe these ancient links in contemporary organisms only as occasional, even though very important elements in their general metabolic network.

At the stage in the development of biological systems at which we have arrived, the sole structural element might have been the protein–lipid membrane. Consequently, definite order in the chemical conversion and in the already fairly complex and multi-link chain of reactions occurring here could be attained mainly by the establishment of strictly co-ordinated relationships between the speeds of the individual reactions forming part of the chain, in the same way as this occurs in Buchner fluid.

Consequently, at this stage there was still no need for very accurate, spatially fixed auto-reproduction of the system. Constancy of its composition was achieved by constancy of the exchange network, the repeatability of its chains and cycles leading to fresh formation in the process of synthesis of almost the same substances of the growing system. Multiplication of these systems could be achieved by simple partition resulting from the action of external mechanical agencies.

But it is quite understandable that, with such a form of organization, the longer and more branched the chain of metabolism and the greater the number of

reactions entering into it, the stricter had to be the co-ordination of their speeds and the more perfect the mechanisms required to attain this. Consequently, the few and poorly specialized catalyst co-enzymes previously existing proved inadequate for this complex task and the progressive evolution of the biological systems proceeded towards the creation of a whole arsenal of powerful new catalyst enzymes.

The striking correspondence between their intramolecular structure and the biological functions performed by them, which is an essential feature of contemporary enzymes, could only have arisen in a process of selection of the evolving systems. Finally, the protein-like substances with no specific arrangement of the amino acid radicals in the polypeptide chain and a more or less chance architectonic structure of the macromolecule, which entered into the composition of the original coacervate droplets or were formed there during subsequent syntheses, were completely devoid of catalytic activity or were very bad catalysts. Of the many variants arising in this way by natural selection the only ones preserved were those whose participation in the metabolism of the given system contributed to its prolonged existence, growth and multiplication.

It must be realized clearly that, between the original protein-like catalyst and the very perfectly constructed contemporary enzyme, there have been tested and rejected in the process of evolution not less, and possibly considerably more variant organizations than there have been between the fin of the shark and the human hand.

The perfecting both of the entire living system as a whole and of its individual mechanisms has been going on continuously over a period of many hundreds of millions of years in the process of interaction between biological systems and the external medium and as result of the effect of natural selection. More particularly, both protein enzymes and the mechanisms connected with their synthesis have been perfected and have become more and more adapted to their biological functions.

An overwhelming number of catalytic variants produced in the process of evolution have been irretrievably lost to us, eliminated by natural selection. Consequently, we now extract only enzymes of very perfect structure from contemporary organisms. But perhaps even now, with more careful research, it will be possible to detect some of the evolution of these catalysts. I have already come fairly close in my exposition to the forms of metabolic organization which are peculiar to the contemporary organism most primitive in this respect, namely anaerobes and heterotrophes. It is now possible to trace their further evolution, connected with the development of autotrophy and the capacity for respiration, by comparison of living forms now existing. Much factual material has now been accumulated in scientific literature on this subject. It is hoped that it will be considerably supplemented at our symposium.

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